16:51:37 OCA PAD AMENDMENT - PROJECT HEADER INFORMATION 07/31/91

Active

Project #: G-33-650 Cost share #: Rev #: 10
Center # : R6683-0A0 Center shr #: OCA file #:
Work type : RES
Contract#: DAMD17-89-C-9008 Mod #: ADMIN. Document : CONT
Prime #: Contract entity: GTRC

Subprojects ? : N CFDA: NA
Main project #: PE #: 612787

Project unit: CHEMISTRY Unit code: 02.010.136
Project director(s):
POWERS J C CHEMISTRY (404)894-4038

Sponsor/division names: ARMY / FT DETRICK, MD
Sponsor/division codes: 102 / 004

Award period: 890201 to 910730 (performance) 911231 (reports)

Sponsor amount	New this change	Total to date
Contract value	0.00	327,491.00
Funded	0.00	327,491.00
Cost sharing amount		0.00

Does subcontracting plan apply ?: N

Title: PROTEASE INHIBITORS AS ANTIVESICANTS

PROJECT ADMINISTRATION DATA

OCA contact: William F. Brown 894-4820

Sponsor technical contact

A. J. LIN, PHD.
(301)427-5423

WALTER REED ARMY INSTITUTE OF RES.
DEPT. OF MEDICINAL CHEMISTRY/BLDG.40
WASHINGTON, DC 20307-5100

Sponsor issuing office

BRIAN E. MARTIN/SGRD-RMA-RC
(301)663-7355

US ARMY MED. RESEARCH ACQ. ACTIVITY
FORT DETRICK
FREDERICK, MD 21701-5014

Security class (U,C,S,TS) : U ONR resident rep. is ACO (Y/N): N
Defense priority rating : NONE GOVT supplemental sheet
Equipment title vests with: Sponsor X GIT
SEE ARTICLE G.5 OF CONTRACT
Administrative comments -
ADMIN. REVISION TO CORRECT ENTRIES FOR THE ANNUAL, QUARTERLY AND FINAL
REPORTS IN THE DELIVERABLE SCHEDULE.

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 01/14/92

Project No. G-33-650_____ Center No. R6683-0A0_____

Project Director POWERS J C_____ School/Lab CHEMISTRY_____

Sponsor ARMY/FT DETRICK, MD_____

Contract/Grant No. DAMD17-89-C-9008_____ Contract Entity GTRC

Prime Contract No. _____

Title PROTEASE INHIBITORS AS ANTIVESICANTS_____

Effective Completion Date 910730 (Performance) 911231 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	Y	_____
Final Report of Inventions and/or Subcontracts	Y	_____
Government Property Inventory & Related Certificate	Y	_____
Classified Material Certificate	N	_____
Release and Assignment	Y	_____
Other _____	N	_____

Comments_____

_____

Subproject Under Main Project No. _____

Continues Project No. _____

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	Y
Reports Coordinator (OCA)	Y
GTRC	N
Project File	Y
Other _____	N
_____	N

NOTE: Final Patent Questionnaire sent to PDPI:

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #1

Reporting Period: February 1, 1989 to April 30, 1989

Report Date: May 12, 1989

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
(Started work on March 1, 1989)		
Józef Oleksyszyn	Research Scientist	100%

Approximate Contract Expenditures to April 30, 1989

Personnel	$9,777
Supplies	314
Travel	0
Other	0
Equipment	594

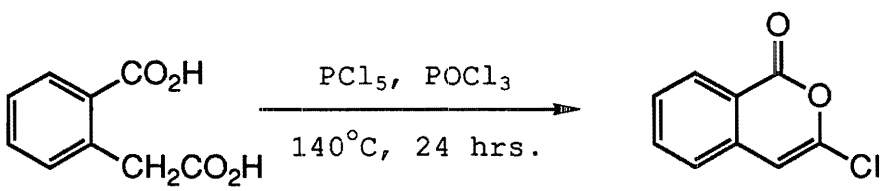
Samples for Submitted for Testing

Twelve compounds have been synthesized this quarter and submitted for testing. In addition, we evaluated the inhibitory potency of some of these compounds *in vitro* with several enzymes including human leukocyte elastase, bovine chymotrypsin, and porcine pancreatic elastase. The compounds will be tested with other enzymes in the future. We plan tests with human skin chymase (a chymotrypsin-like enzyme). Human skin chymases along with other proteases such as human leukocyte elastase, human leukocyte cathepsin G and skin tryptases may be involved in sulfur mustard induced inflammatory lesions. Initial inhibition data are shown in Table I-IV.

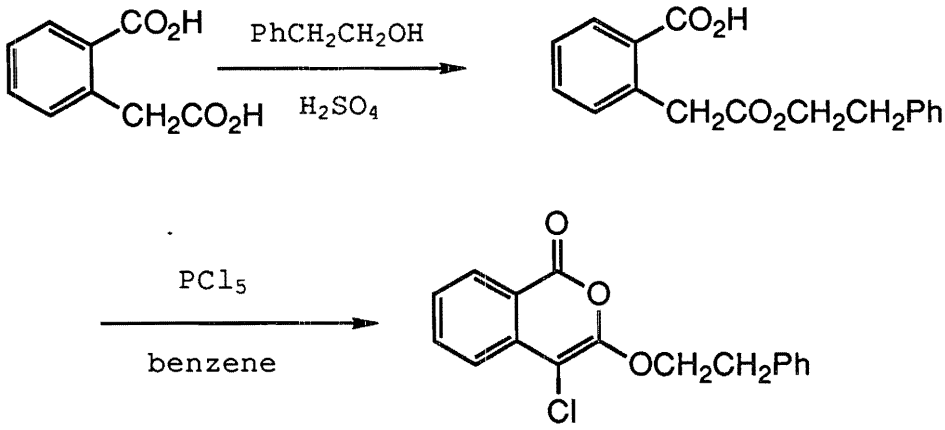
The compounds submitted belong to four classes of inactivators:

Isocoumarin derivatives
Saccharin derivatives
Diphenyl 1-aminoalkylphosphonates derivatives
Miscellaneous inhibitors

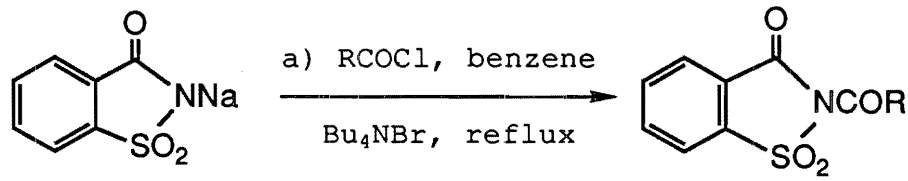
Isocoumarin derivatives. This class of compounds are the most interesting because some of them are potent and specific inhibitors of serine proteases. Two isocoumarin derivatives were synthesized this quarter. The first one, 3-chloroisocoumarin, was obtained directly from homophtalic acid according to a literature procedure, although the yield of this reaction is much lower (3-4%) than that reported in the literature.



The second derivative, 3-chloro-4-(2-phenylethoxy) isocoumarin was synthesized by the method depicted below.

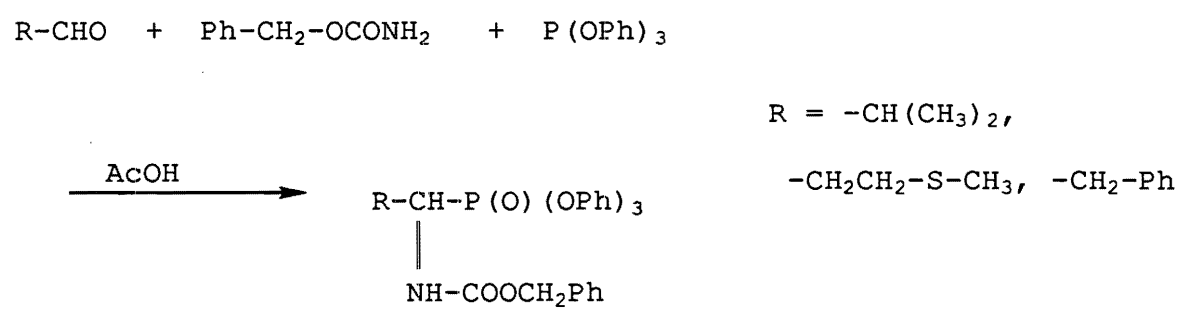


Saccharin derivatives. N-Acyl saccharins have been reported to inhibit a variety of serine proteases and to show selectivity for the different enzymes based on the nature of the acyl group. Therefore we decided to synthesize various N-acyl saccharin derivatives and four have already been submitted. Three of these derivatives were prepared by a procedure different from that used in the literature for the preparation of N-benzoyl and N-furoyl saccharin. In our procedure, tetrabutyl ammonium bromide was used as a phase transfer catalyst

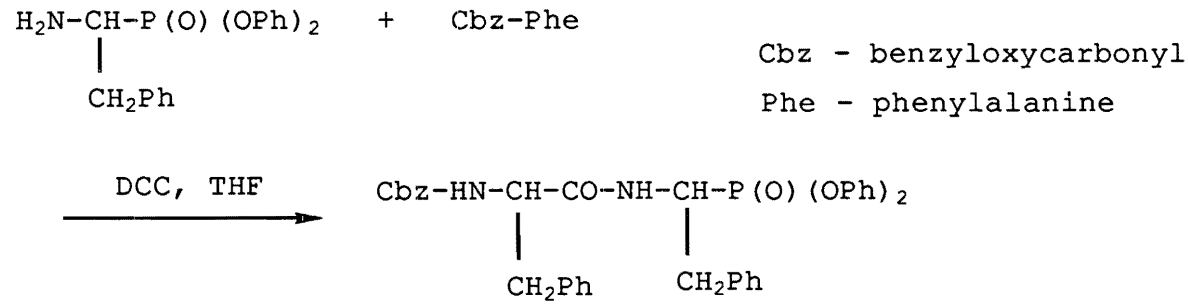


R = Phenyl[a], phenylacetyl[a], 2-furyl[b], diphenylacetyl[a]

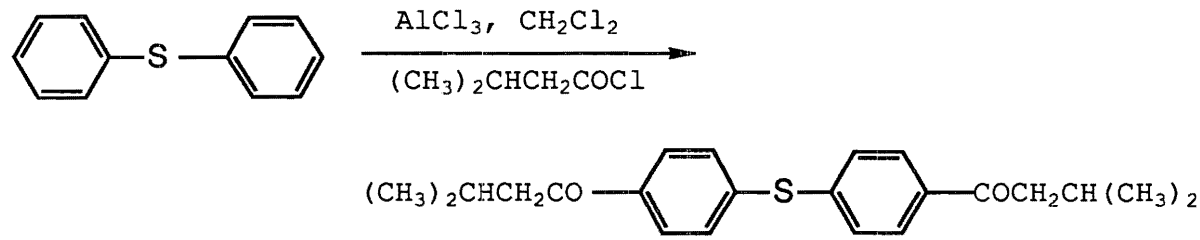
Diphenyl 1-Aminoalkylphosphonates. Recently we discovered that diphenyl esters of 1-aminoalkylphoshonate derivatives are irreversible inhibitors of serine proteases. Three simple carbobenzyloxy derivatives were obtained according to the following literature method.



The dipeptide derivative related to phenylalanine was synthesized using the following procedure.



Miscellaneous Inhibitors. Isatoic anhydride is a general serine protease inhibitor and has also been submitted. The other compound synthesized this quarter was di(*p*-isovaleroylphenyl)sulfide which is a good reversible inhibitor of human leukocyte elastase. The synthesis of this compound by a Friedel-Crafts acylation of diphenyl sulfide is depicted below.



Biological Assays. A few of the compounds have been assayed for activity against a few enzymes. Since this work is incomplete, few correlations can be made at present.

Table I. Inhibition of Various Serine Proteases by Isocoumarin Derivatives.[a]

		Enzyme					
		ChT		PPE		HLE	
Compd. Name	Compd. Num.	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$
3-chloroisocoumarin	II-134 BL57637	9	330	5	510	13	3900
4-chloro-4-(2-phenyl ethoxy)iscoumarin	II-137 BL57413						

[a]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, 2.5% DMSO, pH 7.5 at 25 °C.

Table II. Inhibition of Various Serine Proteases by Diphenyl Esters of 1-Aminoalkylphosphonate Derivatives[a]

		Enzyme					
		ChT		PPE		HLE	
Compd. Name	Compd. Num.	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$
Cbz-Met$^{P}Ph_2$	II-22 BL57959	320	1.6	320	NI	230	0.76
Cbz-Val$^{P}Ph_2$	II-138A BL57968	250	0.4	50	2.5	26	90
Cbz-Phe$^{P}Ph_2$	II-137A BL57422	58	260	58	NI	30	6
Cbz-Phe-Phe$^{P}Ph_2$	II-139 BL57842						

[a]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, 2.5% DMSO, pH 7.5 at 25 °C.

Table III. Inhibition of Various Serine Proteases by 2-Substituted Benzisothiazolinone-1,1-dioxide Derivatives[a]

Benzisothiazolinone-1,1-dioxide		Enzyme					
		ChT		PPE		HLE	
N-Substituent	Compd. Num.	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$
2-Benzoyl	MN-84 BL57977		14,400				
2-Phenylacetyl	MA-89 BL57995		10,800				
2-Diphenylacetyl	MA-87 BL57986		9,960				
2-(2-Furoyl)	MA-86 BL57931						

[a]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, 2.5% DMSO, pH 7.5 at 25 °C.

Table IV. Inhibition of Various Serine Proteases by Miscellaneous Inactivators[a]

Compd. Name	Compd. Num.	Enzyme					
		ChT		PPE		HLE	
		[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$	[I] μM	$k_{obs}/[I]$ $M^{-1}s^{-1}$
Isatoic Anhydride	II-137B BL57646						
Di(4-isovalaroyl phenyl)sulfide	II-145 BL57940						IC_{50} = 2 μm

[a]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, 2.5% DMSO, pH 7.5 at 25 °C.

EXPERIMENTAL SECTION

4-Chloro-3-(2-phenylethoxy)isocoumarin. This compound was prepared according to procedure Harper J. W., and Powers, J. C. (1985) Biochemistry 24, 7200: m.p. 99-100 °C. Anal. Calcd. for $C_{17}H_{13}O_3Cl \cdot 0.5\ H_2O$: C, 65.92; H, 4.52; Cl, 11.46. Found: C, 66.20; H, 4.50; Cl, 11.58.

3-Chloroisocoumarin. This compound was prepared according to the procedure of Davis, W., and Poole, H. G.(1928) J. Chem. Soc. 1616: m.p.98-99 °C. Anal. Calcd. for $C_9H_5O_2Cl$, C, 59.85; H, 2.77; Cl, 19.65. Found, C, 59.74; H, 2.83; Cl, 19.71.

Diphenyl 1-N-benzyloxycarbonylamino-2-phenylethylphosphonate was prepared according to the procedure of Oleksyszyn, J., Subotkowska, L., and Mastalerz, P. (1979) Synthesis 985: m.p. 119-120 °C. Anal. Calcd. for $C_{28}H_{26}O_5NP$: C, 68.94; H, 5.33; N, 2.87. Found: C, 69.00; H, 5.40; N, 2.84.

Diphenyl 1-N-benzyloxycarbonylamino-3-methylthiopropylphosphonate was prepared by the same procedure; m.p. 93-95 °C. Anal. Calcd. for $C_{24}H_{26}O_5NSP$: C, 61.15; H, 5.52; N, 2.97; S, 6.79. Found: C, 61.06; H, 5.60; N, 2.91; S, 6.88.

Diphenyl 1-N-benzyloxycarbonyl-2-methyl-propylphosphonate was prepared by the same procedure; m.p. 104-105 °C. Anal. Calcd. for $C_{24}H_{26}O_5NP$: C,65.56; H, 5.92; N, 3.19. Found: C, 65.67; H, 5.98; N, 3.13.

Diphenyl 1-N-(carbobenzyloxy-phenylalanyl)amino-2-phenylethylphosphonate. To a solution of 2.3 g (6.5 mmol) of diphenyl 1-amino-2-phenylethylphosphonate [Oleksyszyn, J., Subotkowska, L., and Mastalerz, P. (1979) Synthesis 985] and Cbz-Phe-OH 1.95 g (6.5 mmol) in 50 mL of dry THF, 1.32 g (6.6 mmol) of DCC (dicyclohexylcarbodiimide) was added. After 2 days DCU was filtered off and solution was washed with water, 2 times with 50 mL of 5% $NaHCO_3$, water, 2 times with 10% citric acid, water and dried over $MgSO_4$. After evaporation of the solvent, the resulting oil was crystallized from THF-hexane to give 3.5 g (85%) of the dipeptide as a white solid; m.p. 153-155 °C. Anal. Calcd. for $C_{37}H_{35}O_6N_2P$: C, 70.03; H, 5.52; N, 4.42. Found: C, 69.93; H, 5.61; N, 4.51.

Isatoic anhydride. Isatoic anhydride was purchased from Aldrich and recrystallized from ethanol; m.p. 233 °C (decomp). Anal. Cald. for $C_8H_5O_3N$: C, 58.9; H, 3.07; N, 5.59. Found: C, 58.90; H, 3.07; N, 8.52.

Di(4-isovaleroylphenyl)sulfide. A mixture of 10.7 g (80.2 mmol) of $AlCl_3$ in 125 mL of methylene chloride was cooled to

-30 °C under an argon atmosphere. Diphenylsulfide 5.0 g (26.8 mmol) was added followed by isovaleroyl chloride 7.19 mL (59.0 mmol). The solution was warmed to 0 °C and stirred 1.5 hr, then allowed to reach room temperature and stirred overnight. The mixture was poured onto a mixture of ice and concentrated HCl (5:1). The organic layer was extracted with the methylene chloride, washed with 1N NaOH and dried over $MgSO_4$. After removing the solvent, the residue was recrystallized from cyclohexane to give the product as a white solid (89%); m.p. 80-81 °C. Anal. Calcd. for $C_{22}H_{26}O_2S$: C, 74.54; H, 7,39; S, 9.04. Found: C, 74.45; H, 7.42; S, 8.97.

2-Benzoylbenzisothiazolinone-1,1-dioxide. Tetrabutyl ammonium bromide (0.1 g) was added to a suspension of finely ground sodium saccharin (2.0 g, 9.8 mmol) in 30 mL of benzene. Benzoyl chloride (1.2 mL, 10.3 mmol) was added after azeotropic removal of water from this suspension and the resulting mixture was heated to reflux temperature for 3 h. The reaction mixture was filtered while hot, the solid was washed with hot benzene and the filtrate was cooled to 5 °C while scratching the sides of the flask to induce precipitation of the product. The white solid was collected by filtration and recrystallized from benzene/hexane to yield 1.39 g (50%) of pure product; m.p. 162-163 °C (Lit. 160-163 °C). ^{1}H NMR ($CDCl_3$) δ: 8.14 (d, 1H); 8.01 (t, 2H); 7.93 (m, 1H); 7.78 (d of d, 2H); 7.67 (t, 1H); 7.51 (t, 2H). Anal. Calcd. for $C_{14}H_9NO_4S$: C, 58.53; H, 3.16; N, 4.88. Found: C, 58.66; H, 3.19; N, 4.92.

2-(2-Furoyl)benzisothiazolinone-1,1-dioxide. 2-Furoyl chloride (7.4 mL, 75 mmol) was added to a suspension of finely ground sodium saccharin in 40 mL THF and the resulting mixture was heated to reflux temperature for 4 h. The reaction mixture was filtered and the filtrate was concentrated to dryness to give a white solid that was recrystallized from ethyl acetate. The yield of pure product was 4.79 g (35%); m.p. 167-168 °C (Lit. 133-135 °C). ^{1}H NMR ($CDCl_3$) δ: 8.19 (d, 1H); 7.99 (d, 2H); 7.93 (m, 1H); 7.73 (d, 1H); 7.58 (d, 1H); 6.66 (d of d, 1H). Anal. Calcd. for $C_{12}H_7NO_5S$: C, 51.98; H, 2.55; N, 5.05. Found: C, 51.83; H, 2.52; N, 5.07.

2-Phenylbenzisothiazolinone-1,1-dioxide. Tetrabutyl ammonium bromide (0.1 g) was added to a suspension of finely ground sodium saccharin (8.0 g, 39 mmol) in 50 mL of benzene. Phenylacetyl chloride (7.7 mL, 58 mmol) was added to this suspension after azeotropic removal of water and the resulting mixture was heated at reflux temperature for 4 h. The reaction mixture was filtered while hot and the solid was washed with hot benzene. Hexane was added to the filtrate and the solid that resulted was filtered and recrystallized from benzene/hexane to yield 5.52 g (47%) of pure product; m.p. 177-179 °C. ^{1}H NMR ($CDCl_3$) δ: 8.17 (d of d, 1H); 7.99-7.87 (m, 3H); 7.38-7.28 (m, 5H);

4.39 (s, 2H). Anal. Calcd. for $C_{15}H_{11}NO_4S$: C, 59.79; H, 3.68; N, 4.65. Found: C, 59.71; H, 3.73; N, 4.61.

2-Diphenylacetylbenzisothiazolinone-1,1-dioxide. Thionyl chloride (7 mL, 100 mmol) was added to a solution of diphenylacetic acid (10 g, 50 mmol) in 50 mL of benzene and the resulting mixture was heated to reflux temperature for 1 h. Excess thionyl chloride and benzene were removed under reduced pressure (1 mm Hg) and then tetrabutyl ammonium bromide (0.1 g), fresh benzene (50 mL) and finely ground sodium saccharin (6.8 g, 30 mmol) were added in one portion. The resulting mixture was heated to reflux temperature for 4 h and was then filtered while hot. The white solid that was collected was washed with hot benzene and the filtrate was concentrated to a small volume (ca. 10 mL). The white solid that came out of solution was collected by filtration and recrystallized from benzene/hexane to yield 5.43 g (48%) of pure product; m.p. 195-197 °C. ^{1}H NMR ($CDCl_3$) δ: 8.08 (d, 1H); 7.95 (d, 2H); 7.89-7.85 (m, 2H); 7.38-7.27 (m, 10H); 6.30 (s, 1H). Anal. Calcd. for $C_{21}H_{15}NO_4S$: C, 66.83; H, 4.01; N, 3.71. Found: C, 66.89; H, 4.01; N, 3.66.

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #2

Reporting Period: May 1, 1989 to July 31, 1989

Report Date: August 7, 1989

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Józef Oleksyszyn	Research Scientist	100%

Approximate Contract Expenditures to July 31, 1989

Personnel	$19,763
Fringe	2,513
Supplies	2,825
Travel	0
Equipment	595
Overhead	15,193
Total	$40,889

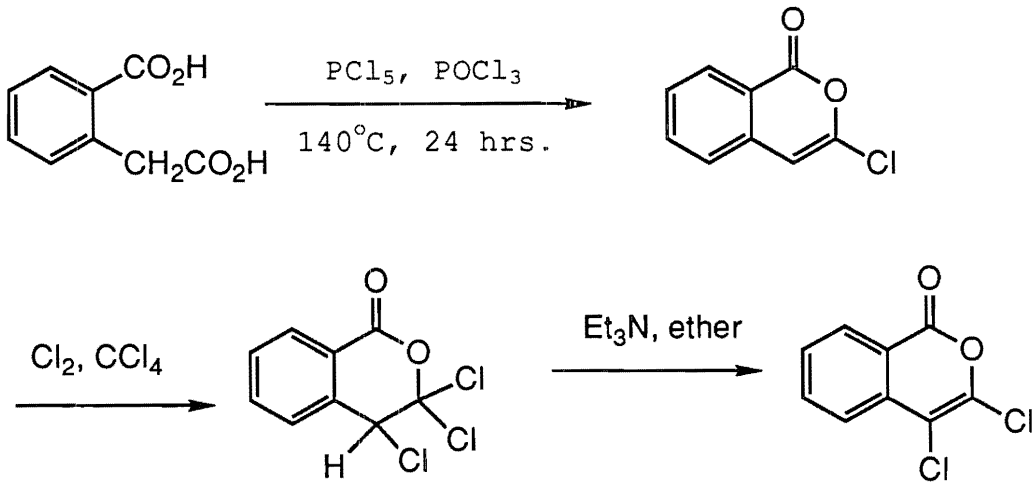
Samples for Submitted for Testing

Three compounds have been synthesized this quarter and submitted for testing. In addition, a number of other compounds are almost ready to submit. We have evaluated the inhibitory potency of many of these compounds *in vitro* with several enzymes including human leukocyte elastase, bovine chymotrypsin, porcine pancreatic elastase, rat mast cell protease II, and human cathepsin G. The compounds will be tested with other enzymes in the future. We plan tests with human skin chymases (chymotrypsin-like enzymes) and human skin tryptases (trypsin-like enzymes). Human skin chymases along with other proteases such as human leukocyte elastase, human leukocyte cathepsin G and skin tryptases may be involved in sulfur mustard induced inflammatory lesions. Inhibition data are shown in Table I-III.

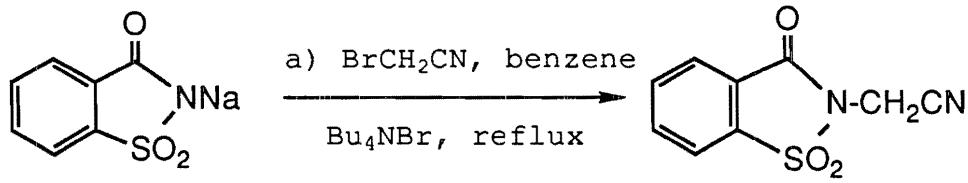
The compounds submitted belong to three classes of inactivators:

Isocoumarin derivatives
Saccharin derivatives
Diphenyl 1-aminoalkylphosphonates derivatives

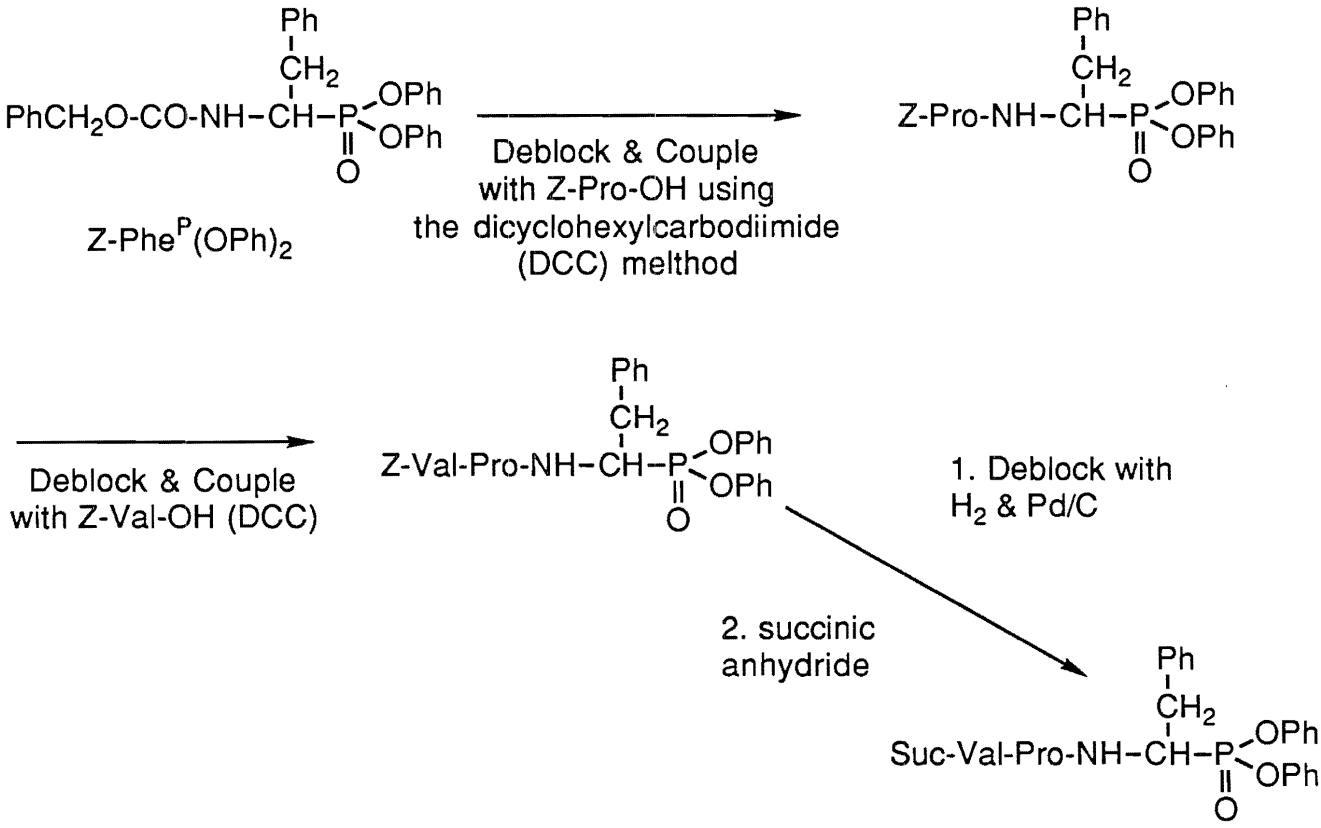
Isocoumarin derivatives. This class of compounds are the most interesting because some of them are potent and specific inhibitors of serine proteases. The general serine protease inhibitor 3,4-dichloroisocoumarin was submitted this quarter. This was prepared by a literature procedure, but in very low yields. Considerable effort was devoted to the synthesis of a 3 g sample.



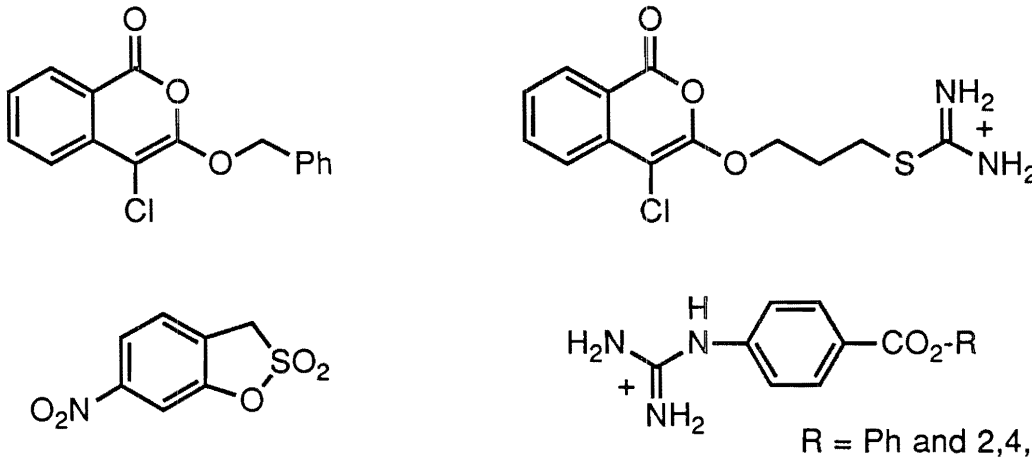
Saccharin derivatives. N-Acyl saccharins have been reported to inhibit a variety of serine proteases and to show selectivity for the different enzymes based on the nature of the acyl group. Several saccharins were submitted last quarter and one additional saccharin, N-cyanomethyl, was submitted this quarter.



Diphenyl 1-Aminoalkylphosphonates. Recently we discovered that diphenyl esters of 1-aminoalkylphoshonate derivatives are irreversible inhibitors of serine proteases. Three simple carbobenzyloxy derivatives were submitted last month and we have now submitted the tripeptide derivative Suc-Val-Pro-$Phe^{P}(OPh)_2$. This derivative has turned out to be one of the most potent inhibitors of chymotrypsin, cathepsin G, and rat mast cell protease II.



Planned Syntheses. A few of the structures which we plan to submit in the near future are shown below.



R = Ph and 2,4,6-trichlorophenyl

Biological Assays. Many of the compounds have been assayed for activity against several enzymes. The results are showed in Tables I-III. At present, no animal studies have been received. We plan to submit a variety of different serine protease inhibitors until we obtain animal test data.

Table I. Inactivation Rates of Various Chymases by Substituted Isocoumarins, Phosphonate Derivatives and other Type of Inhibitors[a].

Inhibitors	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
	Chymotrypsin[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase	Dog Skin Chymase
3,4-dichloroisocoumarin[e] (**1**)	570	28	580	27	82
3-chloroisocoumarin[e] (**2**)	330	24	85		
4-chloro-3-(2-phenylethoxy)-isocoumarin (**3**)	3800	103	200		
isatoic anhydride (**4**)	580	114	250		
di(4-isovalaroylphenyl)-disulfide (**5**)		NI[f]	25%[g]		
Z-Met^{P}(OPh)$_2$ (**6**)	1.6	1.1	24		
Z-Val^{P}(OPh)$_2$ (**7**)	0.4	NI	NI		
Z-Phe^{P}(OPh)$_2$ (**8**)	260	76	89		
Z-Phe-Phe^{P}(OPh)$_2$ (**9**)	7.5	NI	18		

Suc-Val-Pro-Phe$^{P}(OPh)_2$ (**10**)	41,000	36,000	14,700

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[a]Inactivation rates were measured by incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO and at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6μM; cathepsin G, 1.6 μM; RMCPII, 38 nM. Chymotrypsin and cathepsin G were assayed with Suc-Val -Pro-Phe-NA (0.5 mM), and RMCPII were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33mM).

[b]Inhibitor concentrations were the following: **3**, 19.3 μM; **4**, 25 μM, **6**, 320 μM; **7**, 250 μM; **8**, 58 μM; **9**, 64 μM; **10**, 5.2 μM.

[c]Inhibitor concentrations were the following: **3**, 19.3 μM; **4**, 25 μM, **5**, 158 μM; **6**, 128 μM; **7**, 148 μM; **8**, 82 μM; **9**, 64 μM; **10**, 8.4 μM.

[d]Inhibitor concentrations were the following: **3**, 20 μM; **4**, 25 μM; **6**, 128 μM; **7**, 148 μM, **8**, 82 μM; **9**, 64 μM; **10**, 3 μM.

[e]Data was obtained from Harper, et al., (1985) Biochemistry 24, 1831-1841.

[f]No inhibition.

[g]Inhibition was not time dependent, and % inhibition was obtained at 92 μM.

Table II. Inactivation Rates of Various Chymases by Saccharins[a].

Inhibitors	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
	Chymotrypsin[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase	Dog Skin Chymase
N-benzoyl saccharin (**11**)	14,400	45,400	16,000		
N-phenylacetyl saccharin (**12**)	10,800	30,900	1,270		
N-diphenylacetyl saccharin (**13**)	9,960	13,800	9,800		
N-furoyl saccharin (**14**)	22,000	38,500	19,600		
N-cyanomethyl saccharin (**15**)	N.I.				

[a]Inactivation rates were measured by incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO and at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6μM; cathepsin G, 0.8-1.6 μM. Chymotrypsin and cathepsin G were assayed with Suc-Val - Pro-Phe-NA (0.5 mM).

[b]Inhibitor concentrations were the following: **11**, 8.5 μM; **12**, 12.5 μM, **13**, 8.6 μM; **14**, 7.5 μM; **15**, 1 mM.

[c]Inhibitor concentrations were the following: **11**, 7 μM; **12**, 7.2 μM, **13**, 7.7 μM; **14**,.4.8 μM.

[d]Inhibitor concentrations were the following: **11**, 9 μM; **12**, 83 μM, **13**, 13.1 μM; **14**,.9.8 μM.

Table III. Inactivation Rates of Elastases by Substituted Isocoumarins, Phosphonate Derivatives, and other Types of Inhibitors[a].

Inhibitors	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	
	PPE[b]	HLE[c]
3,4-dichloroisocoumarin[d] (**1**)	2,500	9,000
3-chloroisocoumarin[d] (**2**)	510	3,900
4-chloro-3-(2-phenylethoxy)-isocoumarin (**3**)		
isatoic anhydride (**4**)		
di(4-isovalaroylphenyl)-disulfide (**5**)		2 μM[e]
Z-Met^{P}(OPh)$_2$ (**6**)	NI[f]	0.8
Z-Val^{P}(OPh)$_2$ (**7**)	2.5	90
Z-Phe^{P}(OPh)$_2$ (**8**)	NI	6
Z-Phe-Phe^{P}(OPh)$_2$ (**9**)	NI	NI
Suc-Val-Pro-Phe^{P}(OPh)$_2$ (**10**)	NI	NI

[a]Inactivation rates were messured by incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO and at 25 °C. Enzyme concentrations were: PPE, 1.6 μM; HLE, 0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were the following: **6**, 320 μM; **7**, 50 μM; **8**, 58 μM; **9**, 64 μM; **10**, 3 μM.

[c]Inhibitor concentrations were the following: **6**, 230 μM; **7**, 26 μM; **8**, 30 μM; **9**, 35 μM; **10**, 2 μM.

[d]Data was obtained from Harper, et al., (1985) Biochemistry 24, 1831-1841.

[e]IC_{50} was obtained.

[f]No inhibition.

EXPERIMENTAL SECTION

Z-Pro-Phe^P(OPh)$_2$. Z-Pro-OH (1.5 g, 6 mmol) was dissolved in 60 mL of dry THF and cooled to 0 °C. Diphenyl α-amino-2-phenylethylphosphonate (2.1 g, 6 mmol) and DCC (1.22 g, 6 mmol) were added to this solution. The reaction mixture was stirred for 6 h at 0 °C and overnight at room temperature. The precipitated DCU was removed by filtration and 50 mL of ethyl acetate was added to the filtrate. The solution was washed twice with succesive portions of 10% citric acid, water, 4% sodium bicarbonate and water. The resulting solution was dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo* to a residue that was dissolved in 10 mL CH_2Cl_2. The small amount of DCU that came out of solution was removed by filtration and 30 mL of pentane was added to the filtrate to effect crystallization of the product. After a few days the product was filtered and recrystallized from CH_2Cl_2-Hexane to yield 1.86 g (53%) of pure product as a white solid; m.p.: 81-84 °C. Anal. Calcd. for $C_{33}H_{33}O_6N_2P$: C, 67.81; H, 5.65; N, 4.79. Found: C, 67.56; H, 5.79; N, 4.72.

Suc-Val-Pro-Phe^P(OPh)$_2$. Palladium on charcoal (0.1 g, 5% catalyst) was added to a solution of 1.17 g (2 mmol) of Z-Pro-Phe^P(OPh)$_2$ in 50 mL of methanol and the resulting mixture was stirred under an atmosphere of hydrogen at room temperature for 2 h. The reaction mixture was filtered through Celite and Z-Val-OH (0.5 g, 2 mmol) was added to the filtrate. The solvent was removed *in vacuo* and the residue was dissolved in 60 mL of dry THF. DCC (0.4 g, 2 mmol) was added to this solution and the resulting mixture was kept for 6 h at 0 °C and overnight at room temperature. The resulting DCU was removed by filtration and the filtrate was washed with water, twice with 4% $NaHCO_3$, water and twice with 10% citric acid and water. After drying over $NaSO_4$, the solvent was removed *in vacuo* and the oil that resulted was dried under low pressure for a few hours. The dry semisolid was dissolved in 60 mL of ethyl acetate and succinic anhydride (0.2 g, 2 mmol) was then added. After addition of 0.1 g of 5% Pd/C the resulting mixture was stirred under a hydrogen atmosphere until TLC indicated only one new spot. The catalyst was then removed by filtration through Celite and the filtrate was washed several times with water. The resulting solution was dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo* to yield 0.9 g (65%) of product as a hygroscopic solid; m.p.: 50-53 °C. ^{31}P NMR δ: 19.75; 19.23 ppm. Anal. Calcd. for $C_{34}H_{40}O_3N_3P \cdot 2H_2O$: C, 59.56; H, 6.42. Found: C, 59.59; H, 6.42.

2-Cyanomethyl Benzisothiazolinone-1,1-dioxide. Sodium saccharin (5.0 g, 24 mmol) was suspended in 50 mL of

benzene and tetrabutyl ammonium bromide (20 mg) was then added. Water was removed azeotropically from this mixture by heating the suspension to reflux temperature for 45 min. After removal of water, bromoacetonitrile (1.8 mL, 25.6 mmol) was added and the reaction mixture was heated to reflux temperature for 14 h. The mixture was filtered while hot and the solid was washed with hot benzene (5 x 5 mL). The filtrate was concentrated *in vacuo* to a minimum volume (c.a. 5 mL) and hexane was then added to effect complete precipitation of the product. After one recrystallization from chloroform, the yield of pure product was 2.15 g (39.6%); m.p.: 134-5 °C. ^{1}H NMR ($CDCl_3$) δ: 8.17 (d of d, 1H); 8.02-7.89 (m, 3H); 4.63 (s, 2H). Anal. Calcd. for $C_9H_6N_2O_3S$: C, 48.64; H, 2.72; N, 12.61. Found: C, 48.73; H, 2.76; N, 12.52.

G-33650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #3

Reporting Period: August 1, 1989 to October 31, 1989

Report Date: November 10, 1989

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

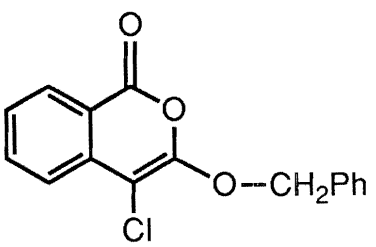
James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Józef Oleksyszyn	Research Scientist	100%

Approximate Contract Expenditures to October 31, 1989

Personnel	$39,647
Fringe	4,602
Supplies	7,315
Travel	1,561
Equipment	595
Overhead	16,300
Total	$70,020

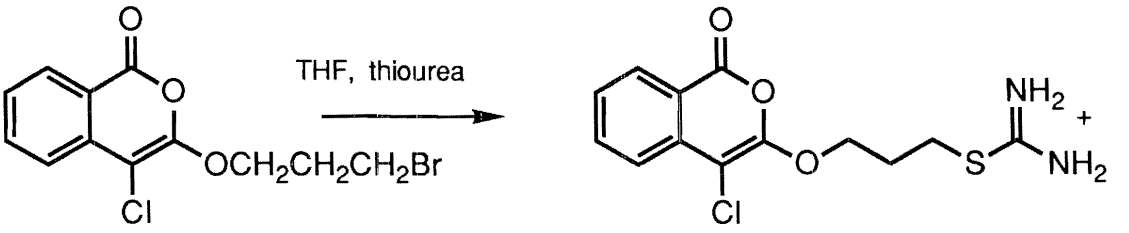
Summary. Seven new compounds have been synthesized this quarter and submitted for testing as antivesicants. The compounds belong to four classes of inactivators: a) isocoumarin derivatives, b) benzoxazinones, c) 5-nitro-3H-1,2-benzoxathiole-2,2-dioxides, and d) derivatives of *p*-guanidinobenzoic acid. The inhibitory potency of many of these compounds along with previously submitted compounds has been evaluated *in vitro* with several serine proteases including a number of chymases, elastases, and tryptases. Inhibition data is described in the section on biological activity and Tables I-III. Additional enzymes and inhibitors will be assayed in the future. We now have available very potent general serine proteases inhibitors, and specific inhibitors for chymases, elastases, and tryptases.

Isocoumarin derivatives. We have synthesized many isocoumarin derivatives in the course of previous research and most of them have been assayed for activity against several enzymes. On the basis of kinetic data some of the best coumponds were chosen, synthesized, and submitted for testing. One of the best inhibitors for chymotrypsin-like enzymes is 3-benzyloxy-4-chloroisocoumarin.



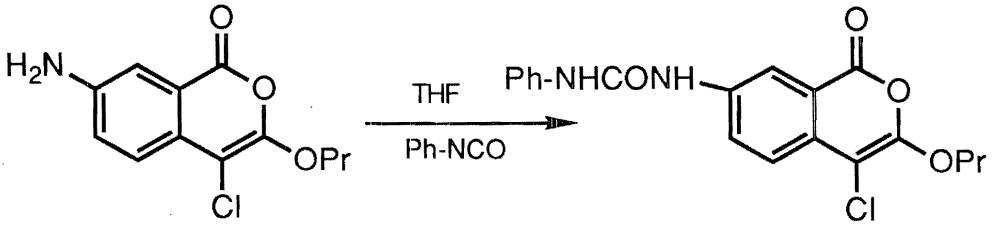
3-benzyloxy-4-chloroisocoumarin

For trypsin-like enzymes one of the best isocoumarin inhibitors is 4-chloro-3-(3-isothiureidopropoxy)isocoumarin obtained by S-alkylation of thiourea with 3-(3-bromopropoxy)-4-chloroisocoumarin.



4-chloro-3-(3-isothiureidopropoxy)isocoumarin

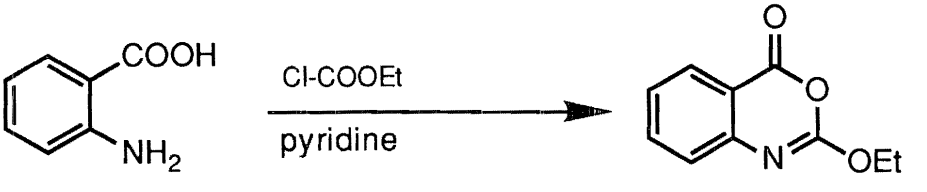
The reaction of phenylisocyanate with 7-amino-4-chloro-3-propoxyisocoumarin afforded one of the best inhibitors for human leukocyte elastase.



4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin

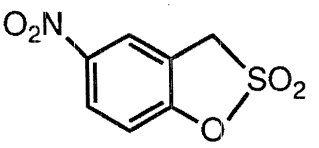
The starting material for this reaction is obtained by a multistep procedure involving nitration of homophthalic acid, esterification of the resulting nitrophthalic acid to the monoester and cyclization of the later to 7-nitro-4-chloro-3-alkoxyisocoumarin using phosphorus pentachloride. The last step, reduction of the nitro group to the amino group can be done only on a small scale (about 1 g), probably due to reaction of the amino group with the isocoumarin ring. In order to obtain a 3 g sample of the inhibitor, we had to repeat this reaction several times.

Benzoxazinones. Several benzoxazinones have been reported to inhibit human leukocyte elastase (HLE) and other serine proteases. This quarter we submitted 2-ethoxy-4H-3,1-benzoxazin-4-one which was synthesized by the following scheme. It is one of the best benzoxazinone inhibitors of HLE.



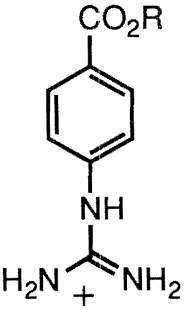
2-Ethoxy-4H-3,1-benzoxazin-4-one

5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide. This sultone is a probably a general inhibitor of serine proteases, but only a few enzymes have been tested at present.



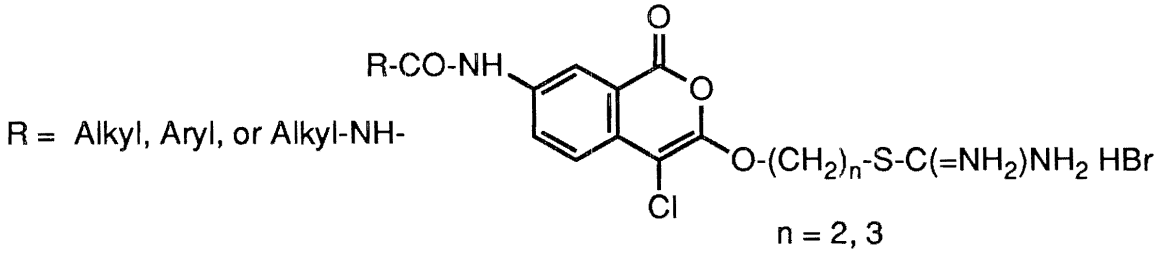
5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide

Derivatives of *p*-Guanidinobenzoic Acid. The cationic *p*-guanidinobenzoic acid is a weak competitive inhibitor of trypsin-like enzymes. Its ethyl ester is an active site titrant for trypsin-like enzymes. Samples of *p*-guanidinobenzoic acid and its ethyl ester were prepared by a previously reported multistep procedure and were submitted this quarter.

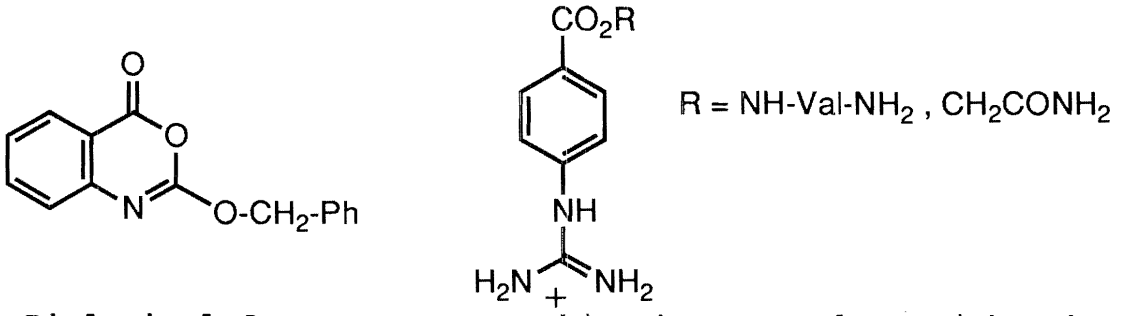


R = H, Et

Planned Syntheses. Recently we have synthesized a series of isocoumarin derivatives containing the isothiureido moiety. They are excellent inhibitors for trypsin-like enzymes. We are presently measuring their inhibitory potency against human lung tryptase and rat skin tryptase. A few of the most active compounds will be synthesized in large amounts and submitted in the near future.



Benzoxazinone analogs resembling the best isocoumarin derivatives for chymotrypsin-like enzymes will also be synthesized (below left). Coupling of *p*-guanidino benzoic acid with valine amide and chloroacetamide has been accomplished and 3 g samples will soon be ready for submission (below right).



Biological Assays. Human skin chymases along with other serine proteases such as human leukocyte elastase, human leukocyte cathepsin G and skin tryptases are thought to be major mediators of sulfur mustard induced inflammatory lesions. All the compounds which we have submitted are listed in Tables I-III along with their inhibitory potency toward a number of serine proteases. The data with bovine chymotrypsin, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The data with porcine pancreatic elastase and human leukocyte elastase is given in Table II. The data with bovine trypsin, human lung tryptase, and rat skin tryptase is given in Table III.

The majority of the inhibitors are irreversible inhibitors of the various enzymes and the second order inhibition contants ($k_{obs}/[I]$) are reported in the tables. Several of the inhibitors have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. An inhibition constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing).

This quarter we started testing some of the inhibitors with tryptases and other trypsin-like enzymes, and the data shown in Table III has been measured recently. New compounds and new measurements have been added to the other two tables. There are still many gaps in the various tables. Several of the enzymes are hard to obtain and only the most promising inhibitors have been assayed with the small amounts of available enzyme. We expect to obtain additional supplies of skin enzymes in the near future and will carry out additional assays with these enzymes at that time. At present, no animal test data has been received. We plan to submit a variety of different classes of serine protease inhibitors until we obtain animal test data.

High Priority Compounds for Testing.

General Serine Protease Inhibitors
- 3,4-dichloroisocoumarin
- N-furoylsaccharin

Chymase Inhibitors
- Suc-Val-Pro-Phe^{P}(OPh)$_2$

Elastase Inhibitors
- 2-ethoxy-4H-3,1-benzoxazin-4-one
- 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin
- di(4-isovalaroylphenyl)disulfide

Tryptase Inhibitors
- 4-chloro-3-(3-isothioureido-propyloxy)isocoumarin

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase	Dog Skin Chymase
		Isocoumarin Inhibitors				
BL58572	3,4-dichloroIC[e]	570	28	580	27	82
BL57637	3-chloroIC[e]	330	24	85		
BL57413	4-chloro-3-(2-phenylethoxy)IC	3800	100	200		
BM00482	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	
		Miscellaneous Inhibitors				
BL57646	isatoic anhydride	580	114	250		
BL57940	di(4-isovalaroylphenyl)disulfide		NI[f]	25%[g]		
		Phosphonate Inhibitors				
BL57959	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		
BL57968	Z-Val^{P}(OPh)$_2$	0.4	NI	NI		
BL57422	Z-Phe^{P}(OPh)$_2$	260	76	89		

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase	Dog Skin Chymase
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI	18		
BL59282	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000		
	Saccharin Inhibitors					
BL57977	N-benzoylsaccharin		15,000	45,000	16,000	
BL57995	N-phenylacetylsaccharin		11,000	31,000	1,300	
BL57986	N-diphenylacetylsaccharin		10,000	14,000	9,800	
BL57931	N-furoylsaccharin		22,000	39,000	20,000	
BM00464	N-cyanomethylsaccharin		NI[f]			

[a]Inactivation rates were measured by incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25° C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), and RMCP II was assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

Table I (Continued).

[b]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 19.3 μM; isatoic anhydride, 25 μM; Z-Met^{P}(OPh)$_2$, 320 μM; Z-Val^{P}(OPh)$_2$, 250 μM; Z-Phe^{P}(OPh)$_2$, 58 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 5.2 μM; N-benzoylsaccharin, 8.5 μM; N-phenylacetylsaccharin, 12.5 μM, N-diphenylacetylsaccharin, 8.6 μM; N-furoylsaccharin, 7.5 μM; N-cyanomethylsaccharin, 1 mM.

[c]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 19.3 μM; isatoic anhydride, 25 μM; di(4-isovalaroylphenyl)disulfide, 158 μM; Z-Met^{P}(OPh)$_2$, 128 μM; Z-Val^{P}(OPh)$_2$, 148 μM; Z-Phe^{P}(OPh)$_2$, 82 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 8.4 μM; N-benzoylsaccharin, 7 μM; N-phenylacetylsaccharin, 7.2 μM, N-diphenylacetylsaccharin, 7.7 μM; N-furoylsaccharin, 4.8 μM.

[d]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 20 μM; isatoic anhydride, 25 μM; Z-Met^{P}(OPh)$_2$, 128 μM; Z-Val^{P}(OPh)$_2$, 148 μM, Z-Phe^{P}(OPh)$_2$, 82 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 3 μM; N-benzoylsaccharin, 9 μM; N-phenylacetylsaccharin, 83 μM, N-diphenylacetylsaccharin, 13.1 μM; N-furoylsaccharin, 9.8 μM.

[e]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) *Biochemistry 24*, 1831-1841.

[f]No inhibition.

[g]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
BL58572	3,4-dichloroIC[d]	2,500	9,000
BL57637	3-chloroIC[d]	510	3,900
BL57413	4-chloro-3-(2-phenylethoxy)IC		
BM01096	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
	Miscellaneous Inhibitors		
BL57646	isatoic anhydride		
BL57940	di(4-isovalaroylphenyl)disulfide		2 μM[e]
BM00651	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM00491	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Phosphonate Inhibitors		
BL57959	Z-Met^{P}(OPh)$_2$	NI[f]	0.8
BL57968	Z-Val^{P}(OPh)$_2$	2.5	90
BL57422	Z-Phe^{P}(OPh)$_2$	NI	6
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	NI	NI
BL59282	Suc-Val-Pro-Phe^{P}(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25° C. Enzyme concentrations were: PPE, 1.6 μM; HLE,

Table II (Continued).

0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were as follows: Z-Met^{P}(OPh)$_2$, 320 μM; Z-Val^{P}(OPh)$_2$, 50 μM; Z-Phe^{P}(OPh)$_2$, 58 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 3 μM; 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC, 52 μM.

[c]Inhibitor concentrations were as follows: Z-Met^{P}(OPh)$_2$, 230 μM; Z-Val^{P}(OPh)$_2$, 26 μM; Z-Phe^{P}(OPh)$_2$, 30 μM; Z-Phe-Phe^{P}(OPh)$_2$, 35 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 2 μM.2-ethoxy-4H-3,1-benzoxazin-4-one, 1.8 μM; 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide, 127 μM; 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC, 1.2 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) *Biochemistry 24*, 1831-1841..

[e]Inhibition was not time dependent and the IC_{50} was obtained.

[f]No inhibition.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, and Rat Skin Tryptase by Isocoumarins and Derivatives of *p*-Guanidinobenzoic Acid[a].

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)		
		Bovine Trypsin[b]	Human Lung[c] Tryptase	Rat Skin[d] Tryptase
	Isocoumarin Inhibitors			
BM00642	4-chloro-3-(3-isothioureidopropoxy)isocoumarin	46,000	260,000	650,000
	Guanidinobenzoic Acid Derivatives			
BM01363	*p*-guanidinobenzoic acid			
BM01185	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7

[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM. Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; ethyl *p*-guanidinobenzoate, 0.43 mM.

Table III (Continued).

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 μM; ethyl *p*-guanidinobenzoate, 0.42 mM.

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 μM; ethyl *p*-guanidinobenzoate, 0.45 mM

EXPERIMENTAL SECTION

2-Ethoxy-4H-3,1-benzoxazin-4-one. To a solution of anthranilic acid (6.85 g, 0.05 mol) in dry pyridine (50 ml) at room temperature under anhydrous conditions was added ethyl chloroformate (19.2 ml, 0.2 mol) dropwise over a 15 min period. After stirring for 2 h, the solvent was removed under reduced pressure and the residue was stirred vigrously into 100 ml of ice-cold water. The insoluble solid was filtered, washed with water and air-dried. The crude product was dissolved in ethyl acetate, treated with charcoal and recrystallized to give the benzoxazinone as a white solid (8.5 g, 89%); m.p. 88-90° C. Anal. Calcd. for $C_{10}H_9NO_3$: C, 62.83; H, 4.71; N, 7.33. Found: C, 66.78; H, 4.73; N, 7.31.

5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide was purchased from Eastman Kodak; m.p.: 142-144° C. Anal. Calcd. for $C_7H_5O_5NS$; C, 39.04; H, 2.32; N, 6.51; S, 14.87. Found: C, 39.12; H, 2.33; N, 6.51; S, 14.96.

4-Chloro-3-(3-isothioureidopropoxy)isocoumarin. This inhibitor was prepared as described by Kam, C-M., Fujikawa, K., and Powers, J. C. (1988) *Biochemistry 27*, 2547; m.p. 174-176° C (decomp). Anal. Calcd. for $C_{13}H_{14}BrClO_3N_2S$; C, 39.65; H, 3.56; N, 7.12; S, 8.13. Found: C,39.70; H, 3.62; N, 7.08; S, 8.23.

3-Benzyloxy-4-chloroisocoumarin. This inhibitor was prepared as described by Harper, J. W, and Powers, J. C (1985) *Biochemistry 24*, 7200; m.p. 90-92° C. Anal. Calcd. for $C_{16}H_{11}O_3Cl$; C, 67.02; H, 3.84; Cl, 12.38. Found: C, 66.93; H, 3.92; Cl, 12.28.

4-Chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin. To 3.05 g (0.012 mol) of 7-amino-4-chloro-3-propoxyisocoumarin [Harper, J. W., and Powers, J. C. (1985) *Biochemistry 24*, 7200] in 20 ml of dry THF, 1.44 g (0.012 mol) of phenylisocyjanate was added. The solution was kept at room temperature for a few days and the solid which crystallized out was filtered, washed with pentane, and air-dried; yield 3.3 g (74%), m.p. 235-236° C (decomp). Anal. Calcd. for $C_{19}H_{17}ClO_4N_2$; C, 61.22; H, 4.56; N, 7.52; Cl, 9.52. Found: C, 61.30; H, 4.62; N, 7.51; Cl, 9.60.

***p*-Guanidino benzoic acid.** This compound was prepared according to the procedure of Chase, T., and Shaw E.(1970) *Methods in Enzymol. 19*, 22; m.p. 274° C (dec.). Anal. Calcd. for $C_8H_{10}ClN_3O_2$: C, 44.56; H, 4.67; N, 19.49; Cl, 16.44. Found: C, 44.73; H, 4.65; N, 19.34; Cl, 16.58.

Ethyl *p*-guanidino benzoate. This compound was made according to the procedure of Beyerman, H. C., and Bontekoe, J. S. (1953) *Rec. Trav. Chim. Pays-Bas 72*, 643; m.p. 164-5° C (dec.).

Anal. Calcd. for $C_{10}H_{14}ClN_3O_2$: C, 49.28; H, 5.79; N, 17.24; Cl, 14.55. Found: C, 49.34; H, 5.79; N, 17.17; Cl, 14.52.

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #4

Reporting Period: November 1, 1989 to January 31, 1990

Report Date: February 17, 1990

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

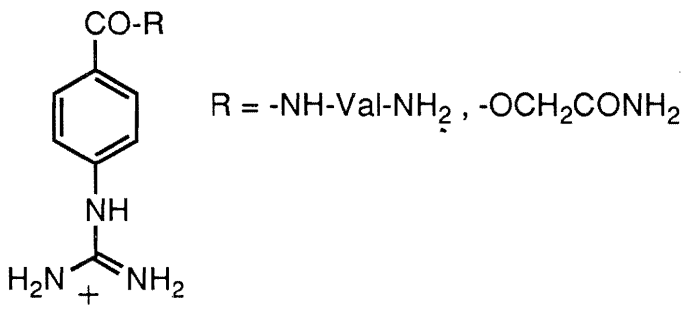
Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Józef Oleksyszyn	Research Scientist	100%

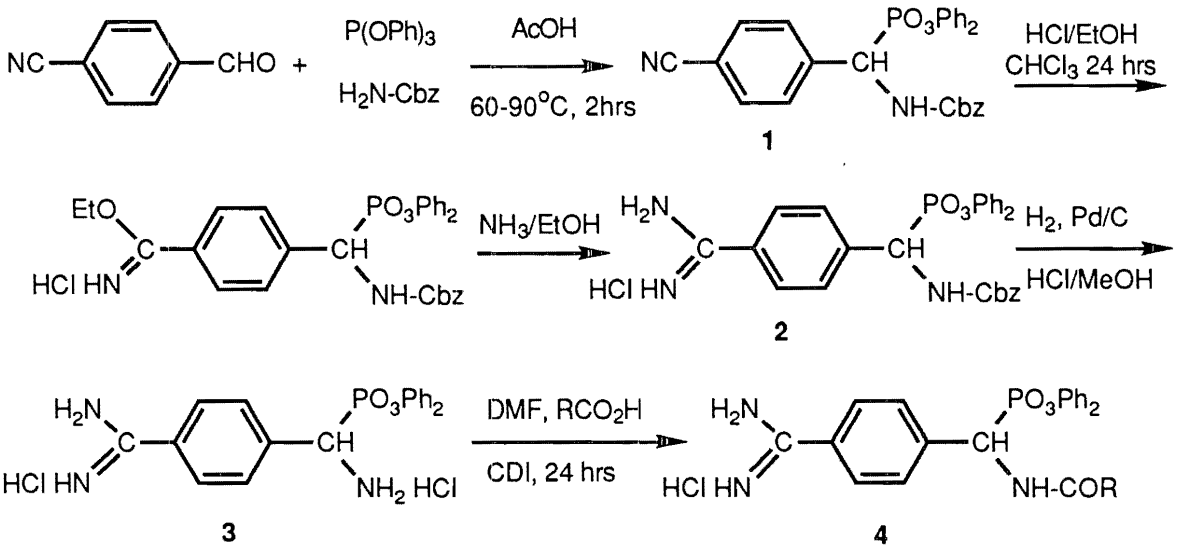
Approximate Contract Expenditures to January 31, 1990

Personnel	$57,526
Fringe	10,046
Supplies	8,089
Travel	1,561
Equipment	595
Overhead	47,930
Total	$125,747

Progress Report. Two new compounds have been synthesized this quarter and submitted for testing as antivesicants. The compounds are N-(*p*-guanidinobenzoyl)glycine amide hydrochloride and N-(*p*-guanidinobenzoyl)valine amide hydrochloride (the structures are shown below).

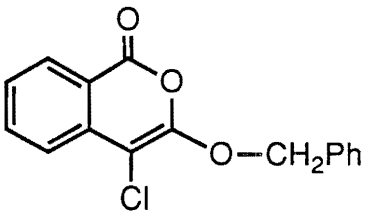


Several N-substituted derivatives of α-amino(4-amidinophenyl)methanephosphonate diphenyl ester have been synthesized on a small scale as possible tryptase inhibitors using the following synthetic scheme.



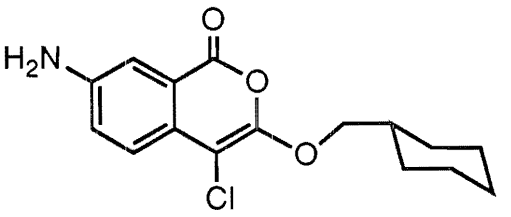
The inhibitory potency of these compounds along with several previously submitted compounds is being evaluated *in vitro* with several trypsin-like enzymes.

Isocoumarin derivatives. We have synthesized many isocoumarin derivatives in the course of previous research and most of them have now been assayed for activity against several chymases. On the basis of kinetic data some of the better coumponds were chosen, synthesized, and submitted for testing. One of the best inhibitors for chymotrypsin-like enzymes is 3-benzyloxy-4-chloroisocoumarin, which was submitted last year.



3-benzyloxy-4-chloroisocoumarin

A logical derivative of this very active analog would posess an amino group at position 7, which would provide a synthetic handle for many other derivatives with improved binding. However,the synthesis of 7-amino-3-benzyloxy-4-chloroisocoumarin failed when no method could be found that would reduce the nitro group without concurrent removal of the benzyloxy functional group or opening of the isocoumarin ring. In order to circumvent this problem we decided to synthesize the 3-cyclohexylmethyloxy derivative (shown below).



The starting isocoumarin is obtained by a multistep procedure involving nitration of homophthalic acid, esterification of the resulting nitrophthalic acid with cyclohexylmethanol to the monoester and cyclization of the later to the 3-alkoxy-4-chloro-7-nitroisocoumarin using phosphorus pentachloride. The last step, reduction of the nitro group to the amino group could be done only on a small scale (about 1 g), probably due to reaction of the amino group with the isocoumarin ring. A 3 g sample will soon be ready for submission.

Biological Assays. Human skin chymases along with other serine proteases such as human leukocyte elastase, human leukocyte cathepsin G and skin tryptases are thought to be major mediators of sulfur mustard induced inflammatory lesions. All the compounds which we have submitted are listed in Tables I-III along with their inhibitory potency toward a number of serine proteases. The data with bovine chymotrypsin, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The data with porcine pancreatic elastase and human leukocyte elastase is given in Table II. The data with bovine trypsin, human lung tryptase, and rat skin tryptase is given in Table III.

The majority of the inhibitors are irreversible inhibitors of the various enzymes and the second order inhibition contants ($k_{obs}/[I]$) are reported in the tables. Several of the inhibitors have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. An inhibition constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing).

This quarter we continued testing some of the inhibitors with tryptases and other trypsin-like enzymes, and the data shown in Table III has been measured recently. New compounds and new measurements have been added to the other two tables. There are still many gaps in the various tables. Several of the enzymes are hard to obtain and only the most promising inhibitors have been assayed with the small amounts of available skin chymase and tryptase. At present, no animal test data has been received. We plan to submit a variety of different classes of serine protease inhibitors until we obtain animal test data.

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Isocoumarin Inhibitors				
BL58572	3,4-dichloroIC[g]	570	28	580	27	82
BL57637	3-chloroIC[g]	330	24	85		
BL57413	4-chloro-3-(2-phenylethoxy)IC	3800	100	200	340	
BM00482	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	
	7-amino-4-chloro-3-cyclohexyloxymethylIC				25	
		Miscellaneous Inhibitors				
BL57646	isatoic anhydride	580	114	250		
BL57940	di(4-isovalaroylphenyl)disulfide		NI[h]	25%[i]		
		Phosphonate Inhibitors				
BL57959	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
BL57968	Z-Val^{P}(OPh)$_2$	0.4	NI[h]	NI[h]		
BL57422	Z-Phe^{P}(OPh)$_2$	260	76	89		
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI[h]	18		
BL59382	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000	190,000	
	Saccharin Inhibitors					
BL57977	N-benzoylsaccharin	15,000	45,000	16,000		
BL57995	N-phenylacetylsaccharin	11,000	31,000	1,300		
BL57986	N-diphenylacetylsaccharin	10,000	14,000	9,800		
BL57931	N-furoylsaccharin	22,000	39,000	20,000		
BM00464	N-cyanomethylsaccharin	NI[h]				

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25°

C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM; human skin chymase, 0.07

Table I (Continued).

μM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), human skin chymase and RMCP II were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 19.3 μM; isatoic anhydride, 25 μM; Z-Met^{P}(OPh)$_2$, 320 μM; Z-Val^{P}(OPh)$_2$, 250 μM; Z-Phe^{P}(OPh)$_2$, 58 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 5.2 μM; N-benzoylsaccharin, 8.5 μM; N-phenylacetylsaccharin, 12.5 μM, N-diphenylacetylsaccharin, 8.6 μM; N-furoylsaccharin, 7.5 μM; N-cyanomethylsaccharin, 1 mM.

[c]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 19.3 μM; isatoic anhydride, 25 μM; di(4-isovalaroylphenyl)disulfide, 158 μM; Z-Met^{P}(OPh)$_2$, 128 μM; Z-Val^{P}(OPh)$_2$, 148 μM; Z-Phe^{P}(OPh)$_2$, 82 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 8.4 μM; N-benzoylsaccharin, 7 μM; N-phenylacetylsaccharin, 7.2 μM, N-diphenylacetylsaccharin, 7.7 μM; N-furoylsaccharin, 4.8 μM.

[d]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 20 μM; isatoic anhydride, 25 μM; Z-Met^{P}(OPh)$_2$, 128 μM; Z-Val^{P}(OPh)$_2$, 148 μM, Z-Phe^{P}(OPh)$_2$, 82 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 3 μM; N-benzoylsaccharin, 9 μM; N-phenylacetylsaccharin, 83 μM, N-diphenylacetylsaccharin, 13.1 μM; N-furoylsaccharin, 9.8 μM.

[e]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 45 μM; 7-amino-4-chloro-3-cyclohexylmethylIC, 0.44 mM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 0.54 μM;

[g]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) *Biochemistry 24*, 1831-1841.

[h]No inhibition.

Table I (Continued).

[i]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
BL58572	3,4-dichloroIC[d]	2,500	9,000
BL57637	3-chloroIC[d]	510	3,900
BL57413	4-chloro-3-(2-phenylethoxy)IC		
BM01096	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
	Miscellaneous Inhibitors		
BL57646	isatoic anhydride		
BL57940	di(4-isovalaroylphenyl)disulfide		2 μM[e]
BM00651	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM00491	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Phosphonate Inhibitors		
BL57959	Z-Met^{P}(OPh)$_2$	NI[f]	0.8
BL57968	Z-Val^{P}(OPh)$_2$	2.5	90
BL57422	Z-Phe^{P}(OPh)$_2$	NI	6
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	NI	NI
BL59282	Suc-Val-Pro-Phe^{P}(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25° C. Enzyme concentrations were: PPE, 1.6 μM; HLE,

Table II (Continued).

0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were as follows: Z-Met^{P}(OPh)$_2$, 320 μM; Z-Val^{P}(OPh)$_2$, 50 μM; Z-Phe^{P}(OPh)$_2$, 58 μM; Z-Phe-Phe^{P}(OPh)$_2$, 64 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 3 μM; 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC, 52 μM.

[c]Inhibitor concentrations were as follows: Z-Met^{P}(OPh)$_2$, 230 μM; Z-Val^{P}(OPh)$_2$, 26 μM; Z-Phe^{P}(OPh)$_2$, 30 μM; Z-Phe-Phe^{P}(OPh)$_2$, 35 μM; Suc-Val-Pro-Phe^{P}(OPh)$_2$, 2 μM.2-ethoxy-4H-3,1-benzoxazin-4-one, 1.8 μM; 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide, 127 μM; 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC, 1.2 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) *Biochemistry 24*, 1831-1841..

[e]Inhibition was not time dependent and the IC_{50} was obtained.

[f]No inhibition.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins and Derivatives of *p*-Guanidinobenzoic Acid[a].

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Isocoumarin Derivatives					
BM00642	4-chloro-3-(3-isothioureido-propoxy)isocoumarin	46,000	260,000	650,000	83,000	13,000
	Guanidinobenzoic Acid Derivatives					
BM01363	*p*-guanidinobenzoic acid	NI[g]				
BM01185	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7		NI[g]
BM02655	N-(*p*-guanidinobenzoyl) valine amide	4.4	4.7	1.3	2,000	
BM03143	N-(*p*-guanidinobenzoyl) glycine amide	100	19%[h]	130,000	5.8	NI[g]
	Phosphonate Inhibitors					
	Z-NHCH(AmPh)PO_3Ph_2[i]					

Table III (Continued).

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[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM; human r-t-PA, 0.017 μM Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase, human skin tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; *p*-guanidinobenzoic acid, 0.44 mM; ethyl *p*-guanidinobenzoate, 0.43 mM; p-guanidinobenzoyl valine amide,0.44 mM; 2-(*p*-guanidinobenzoyl)acetamide, 0.44 mM.

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 μM; ethyl *p*-guanidinobenzoate, 0.42 mM; *p*-guanidinobenzoyl valine amide, 0.42 mM; 2-(*p*-guanidinobenzoyl)acetamide, 0.42 mM.

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 μM; ethyl *p*-guanidinobenzoate, 0.45 mM ; *p*-guanidinobenzoyl valine amide, 0.42 mM; 2-(*p*-guanidinobenzoyl)acetamide, 0.44 μM.

[e]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.35 μM; *p*-guanidinobenzoyl valine amide, 3.5 μM; 2-(*p*-guanidinobenzoyl)acetamide, 1.7 mM.

Table III (Continued).

[f]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 4.3 μM; ethyl *p*-guanidinobenzoate, 0.44 mM; 2-(*p*-guanidinobenzoyl)acetamide, 0.43 mM.

[g]No inhibition.

[h]Inhibition was not time dependent and the % inhibition was measured at 0.42 mM.

[i] AmPh = 4-amidinophenyl

EXPERIMENTAL SECTION

N-(*p*-Guanidinobenzoyl)glycine Amide Hydrochloride. Water was removed azeotropically from a mixture of *p*-guanidinobenzoic acid hydrochloride(2.51 g, 12 mmol) and N-tetrabutylammonium bromide (100 mg) in 50 mL of benzene. 1,2,2,6,6-Pentamethylpiperidine (PMP) was then added (2.1 mL, 12 mmol), followed by 2-chloroacetamide (1.2 g, 13.2 mmol). The resulting mixture was heated to reflux temperature for 24 hours, it was filtered while hot and the beige solid was throroughly washed with acetone. The crude solid was recrystallized from 95% ethanol to yield 1.06 g (34%) of pure product as a white solid, mp 249-51°C. ^{1}H NMR (d_6-Me_2SO) δ: 8.05 (d,2H); 7.75 (broad s, 3H); 7.58 (broad s, 1H); 7.36 (d,2H); 7.28 (broad s, 1H); 4.66 (s, 2H). Anal. Calcd. for $C_{10}H_{13}ClN_4O_3$: C, 44.04; H, 4.80; N, 20.55; Cl, 13.00. Found: C, 43.91; H, 4.85; N, 20.45; Cl, 12.93.

N-(*p*-Guanidinobenzoyl)valine Amide Hydrochloride. Triethylamine (3.2 mL, 23 mmol) was added to a solution of valine amide hydrochloride (3.1 g, 23 mmol) in 100 mL of DMF. 1-Hydroxybenzotriazole (HOBt, 3.15 g, 23 mmol) was added to this mixture, followed by *p*-guanidinobenzoic acid hydrochloride (5.0 g, 23 mmol) and the reaction mixture was then cooled in an ice bath. N,N-diclyclohexylcarbodiimide (DCC, 5.2 g, 25 mmol) was added to the cold (0-5 °C) solution and the resulting mixture was stirred at 5-10 °C for 24 h. The white solid in suspension (DCU) was filtered and washed with EtOAc. The filtrate was concentrated to dryness *in vacuo* to a foam which was triturated with chloroform (5 x 200 mL) with vigorous mechanical stirring to remove $NEt_3 \cdot HCl$. The resulting amorphous solid was taken up in 100 mL of water, filtered through Celite to remove HOBt and the filtrate was lyophylized to yield 5.13 g (71%) of pure

product as a white amorphous solid. ^{1}H NMR (d_6-Me_2SO) δ: 10.25 (br.s, 1H); 8.22 (d, 1H), 7.98 (d, 2H); 7.68 (br.s, 3H); 7.54 (s, 1H); 7.30 (d, 2H); 7.08 (br.s, 1H); 4.26 (t, 1H); 2.11 (m, 1H); 0.91 (d, 3H); 0.89 (d,3H). Anal Calcd. for $C_{13}H_{20}ClN_5O_2 \cdot 0.8\ H_2O$: C, 47.56; H, 6.63; N, 21.33; Cl, 10.80. Found: C, 47.56; H, 6.52; N, 21.26; Cl, 10.68.

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #5

Reporting Period: February 1, 1990 to April 30, 1990

Report Date: May 11, 1990

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

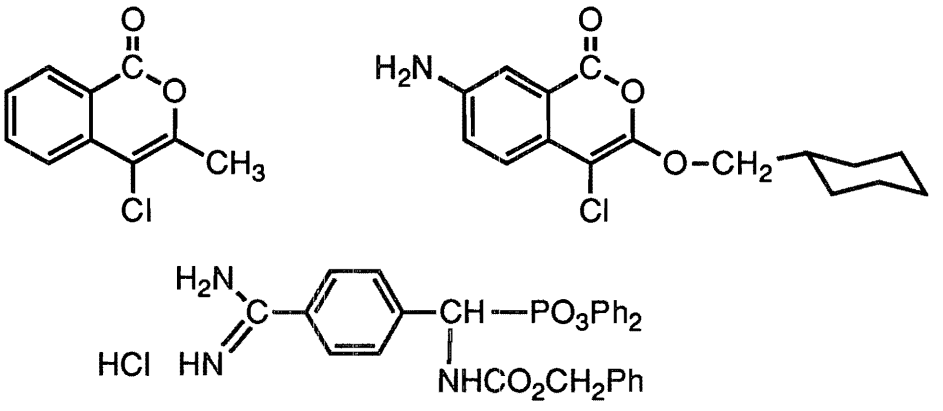
James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Girish S. Patil	Postdoctoral Associate	100%

Dr. Jozef Oleksyszyn left Georgia Tech for a job at Cortec in Denver, CO in early March 1990 and was replaced by Dr. Girish Patil who started in late March.

Approximate Contract Expenditures to April 30, 1990

Personnel	$65,490
Fringe	11,761
Supplies	10,961
Travel	1,561
Equipment	595
Overhead	55,433
Total	$145,801

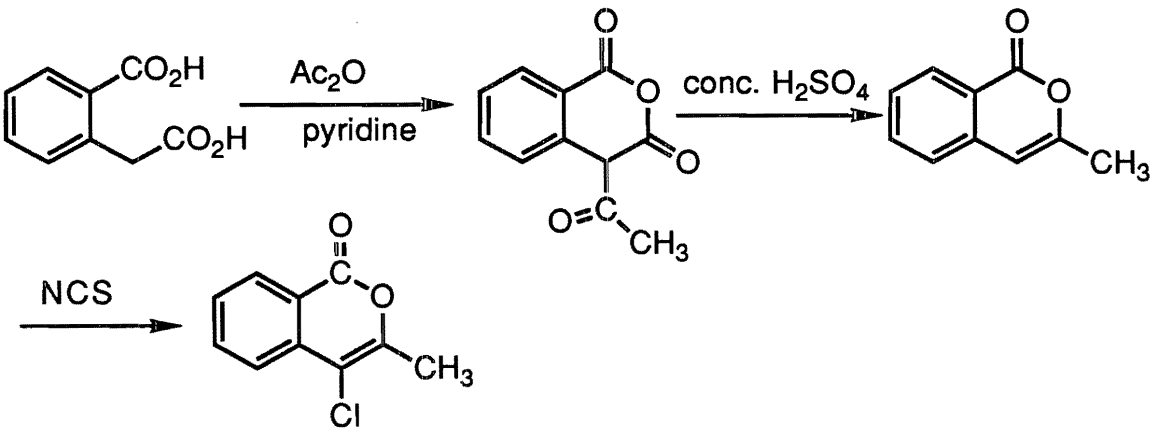
Progress Report. Three new compounds have been synthesized this quarter and submitted for testing as antivesicants. The compounds are 4-chloro-3-methylisocoumarin, 7-amino-4-chloro-3-cyclohexylmethoxyisocoumarin (BM04319) and diphenyl N-benzyloxycarbonyl amino-(4-amidinophenyl)methane phosphonate (BM04328(the structures are shown below).



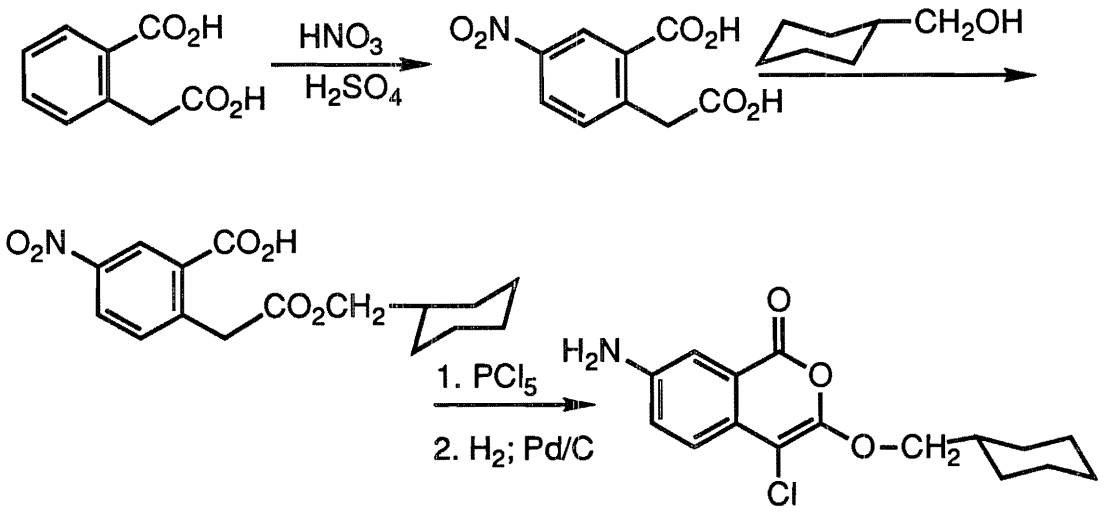
The inhibitory potency of the last two compounds was tested respectively towards trypsin-like enzymes and human skin chymase

and kinetic data were reported in the Annual Report which was recently submitted.

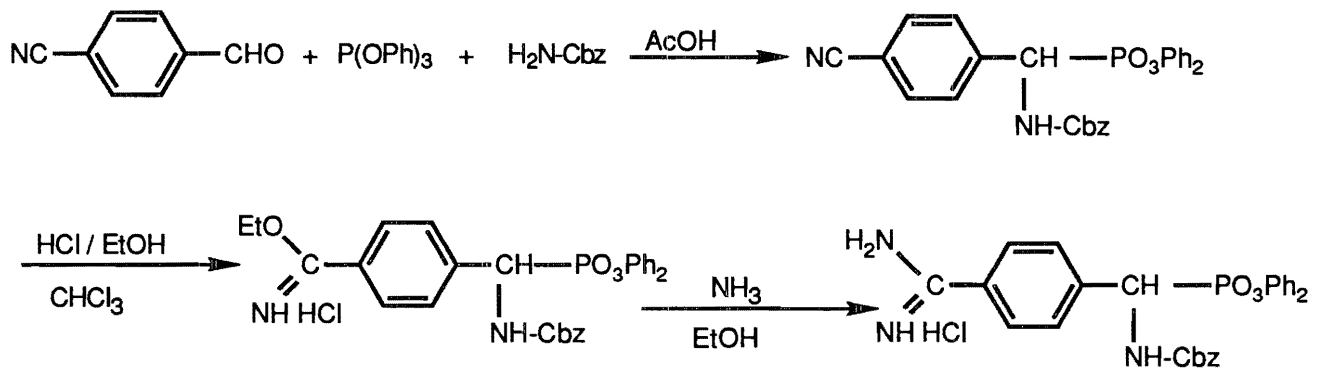
4-Chloro-3-methyl isocoumarin was prepared by reaction of homophthalic acid with acetic anhydride in pyridine to obtain 4-acetyl homophthalic anhydride which was then treated with conc. H_2SO_4 to yield 3-methylisocoumarin. The latter compound gave the final product by reaction with N-chlorosuccinimide. This isocoumarin should be a general protease inhibitor with enhanced stability towards hydrolysis.



7-Amino-4-chloro-3-cyclohexylmethoxyisocoumarin was obtained by a multistep procedure involving nitration of homophthalic acid, esterification of the resulting nitrohomophthalic acid with cyclohexanemethanol to the monoester and cyclization of the latter to the 3-alkoxy-4-chloro-7-nitroisocoumarin using phosphorus pentachloride. The last step, reduction of the nitro group to the amino group can only be done on a small scale (about 1 g), probably due to reaction of the amino group with the isocoumarin ring.



Diphenyl N-benzyloxy carbonylamino(4-amidinophenyl)methane phosphonate was obtained by α-amidoalkylation of triphenyl phosphite using 4-cyanobenzaldehyde and benzyl carbamate to give diphenyl N-benzyloxycarbonylamino(4-cyanophenyl)methane phosphonate. The cyano group was then converted to an imino ether by reaction with HCl in ethanol and finally to an amidino moiety by reaction with ethanolic ammonia.



EXPERIMENTAL SECTION

Cyclohexanemethyl 4-nitro-2-carboxyphenylacetate. A solution containing 10 g (44 mmol) of 5-nitro homophtalic acid (prepared according to H. E. Ungnade, D.V. Nightingale and H. E. French, *J. Org. Chem.* **1945** *10*, 533), cyclohexanemethanol (14 mL, 111 mmol) and 6 drops of conc. H_2SO_4 in 150 mL of benzene was heated to reflux temperature with azeotropic removal of water for 4.5 h. The reaction mixture was cooled to room temperature and then extracted with a saturated solution of $NaHCO_3$ (5 x 25 mL) and water (2 x 25 mL). Excess concentrated HCl was added to the aqueous extract and the white solid which precipitated was collected by filtration and dried to yield 8.58 g (61%) of product that was pure enough to be used in the next step; m.p. 141-2 °C.

4-Chloro-3-cyclohexylmethoxy-7-nitroisocoumarin. A solution of cyclohexanemethyl 4-nitro-2-carboxyphenylacetate (8.58 g, 27 mmol) and PCl_5 (13.9 g, 67 mmol) in 100 mL of benzene was heated to reflux temperature for 4 h and then the solvent was removed *in vacuo*. The residue was recrystallized from 95% EtOH to yield the product as bright yellow needles (5.13 g, 56%); m.p. 102-104 °C.

7-Amino-4-chloro-3-cyclohexylmethoxyisocoumarin. Palladium on charcoal (150 mg, 5% catalyst) was added to a solution of 4-chloro-3-cyclohexylmethoxy-7-nitroisocoumarin (1.24 g, 3.7 mmol) in 200 mL of EtOAc and the resulting mixture was stirred under a hydrogen atmosphere for 6 h at atmospheric pressure. The catalyst was then removed by filtration through a

bed of Celite and the filtrate was concentrated to dryness *in vacuo*. The residue was recrystallized from ethanol/water to yield the product as bright yellow needles (801 mg, 71%); m.p. darkens >180 °C. ^{1}H NMR (d_6-$MeSO_4$) δ: 7.4 (d, 1H), 7.25 (d, 1H), 7.14 (d of d, 1H), 5.76 (br. s, 2H), 4.05 (d, 2H), 1.76-1.58 (m, 6H), 1.30-0.98 (m, 5H). Anal Calcd. for $C_{16}H_{18}ClNO_3$: C, 62.44; H, 5.89; N, 4.55; Cl, 11.52. Found: C, 62.50; H, 5.93; N, 4.51; Cl, 11.42.

Diphenyl N-benzyloxycarbonylamino(4-cyanophenyl)-methanephosphonate. Obtained from 9.75 g 4-cyano-benzaldehyde, 7.65 g benzyl carbamate and 13.5 ml of triphenyl phosphite in 20 ml of glacial acetic acid, according to procedure described earlier (Oleksyszyn J, Subotkowska L, Mastalerz P, *SYNTHESIS*, **1979**,985). Yield 70%; mp. 135-138 °C; Anal. Calcd. for $C_{28}H_{23}O_5N_2P$. $^1/_2$ H_2O: C, 66.27; H, 4.73; N, 5.52. Found; C, 66.03; H, 4.51; N, 5.49.

Diphenyl N-benzyloxycarbonylamino(4-amidinophenyl)-methanephosphonate hydrochloride. A solution of 7 g diphenyl Z-benzyloxycarbonylamino(4-cyanophenyl)-methanephosphonate in 150 mL of dry chloroform and 15 mL of absolute ethanol was saturated with dry HCl at 0 °C. The reaction mixture was kept in the refrigerator until TLC showed no presence of starting material (about 24 h). An excess of pentane was added and the precipitate was removed by filtration and dried under high vacuum. The solid was dissolved in 200 ml of dry methanol and gaseous dry ammonia was bubbled through the solution (one equivalent is required) for approximately 20 min. Methanol and excess amonnia were removed as fast as possible in the rotary evaporator. A portion of fresh

methanol (100 mL) was added and the solution was heated at 50 °C by about 8 h, until TLC shows no presence of imino ether. The solvent was evaporated and the resulting oil was dissolved in chloroform. Addition of ether caused separation of an oil which solidified after a while. The solid that resulted was again dissolved in chloroform, the solution was filtered and the solid product was precipitated using ether. In several experiments the yields were 70-80%; mp. 154-158 °C (decomp); ^{31}P NMR 14,87 ppm. Anal. Calcd. for $C_{28}H_{27}O_5N_3ClP \cdot 0.3NH_4Cl \cdot H_2O$; C, 57.41; H, 5.16; N, 7.52; Cl, 7.31. Found; C, 57.75; H, 5.00; N, 8.86; Cl, 7.43.

Diphenyl amino(4-amidinophenyl)methanephosphonate dihydrochloride. A sample of 1.8 g of diphenyl N-benzyloxy-carbonylamino(4-amidinophenyl)methanephosphonate hydrochloride was dissolved in 150 mL of 2N HCl/methanol solution and after addition of 5% Pd/C catalyst the resulting mixture was stirred under an atmosphere of hydrogen until the theoretical amount of hydrogen was consumed. The catalyst was removed by filtration and after evaporation of methanol a residue was crystallized from ethanol-ether. In several experiments the yields were 60-80%; mp. 213-215 °C; Anal. Calcd. for $C_{20}H_{22}N_3ClP \cdot {}^1/_2H_2O$: C,51.85; H, 4.97; N, 9.08; Cl, 15.34. Found: C, 51.73; H, 5.02; N, 9.10; Cl, 15.36.

4-Chloro-3-methylisocoumarin. To a stirred solution of 10 g (0.06 mol) of 3-methylisocoumarin (Tirodkar,R.B. and Usgaonkar, R. N. *J. Ind. Chem. Soc.* **1969** *46*, 935) in 25 mL of DMF was added 11.16 g (0.08 moles) of N-chlorosuccinimide and the reaction mixture stirred at room temperature overnight. The

reaction mixture was then diluted with ether and the ether layer was washed successively with 10 % HCl, 5 % $NaHCO_3$, water and finally dried over anhydrous $MgSO_4$. Ether was evaporated and the solid obtained was recrystallized from hexane to give 3.5 g (29 %) of the title compound as yellowish needles; m.p. 91-93 ^oC; H^1 NMR ($CDCl_3$) δ: 2.48 (s,3H), 7.55 (m,1H), 7.80 (m,2H), 8.31 (d,1H). Anal. Calcd. for $C_{10}H_7ClO_2$ 0.2 H_2O : C, 60.54; H, 3.53; Cl, 17.91. Found : C, 60.68; H, 3.66; Cl, 17.98.

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #6

Reporting Period: May 1, 1990 to July 31, 1990

Report Date: August 8, 1990

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

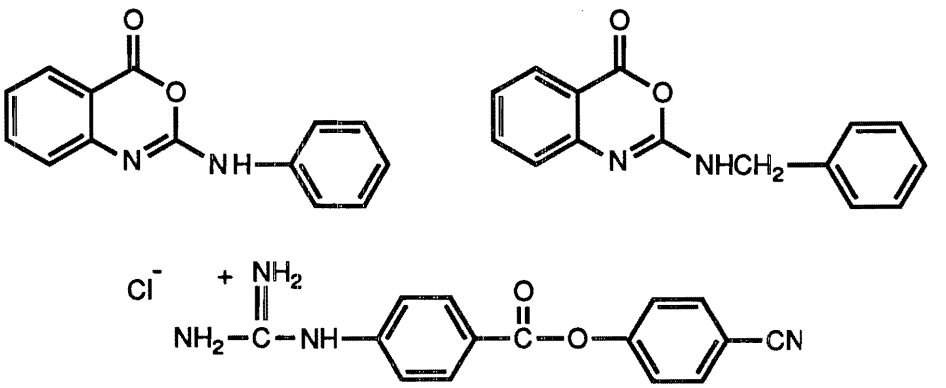
Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Girish S. Patil	Research Scientist	100%

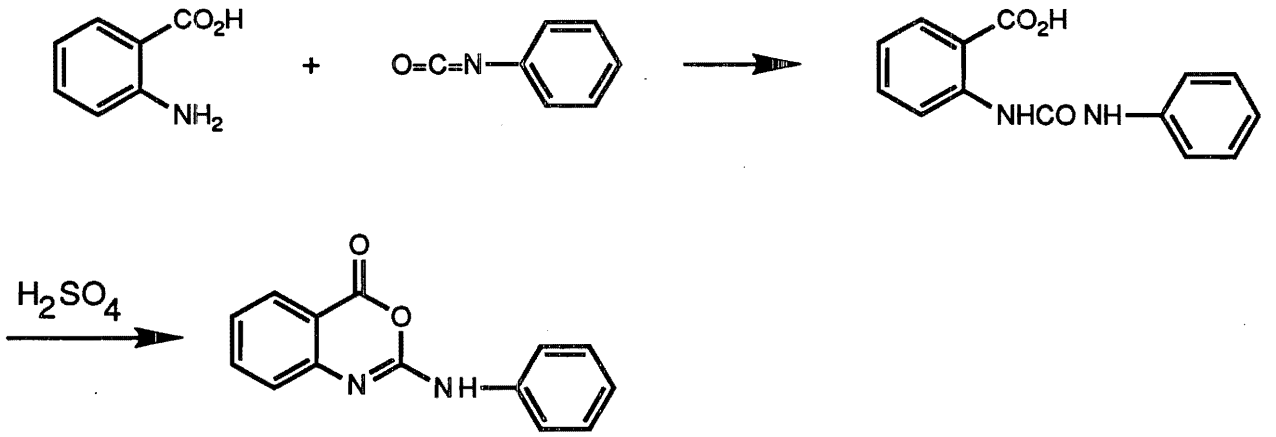
Approximate Contract Expenditures to April 30, 1990

Personnel	$90,316
Fringe	17,288
Supplies	17,283
Travel	2,571
Equipment	595
Overhead	79,468
Total	$207,521

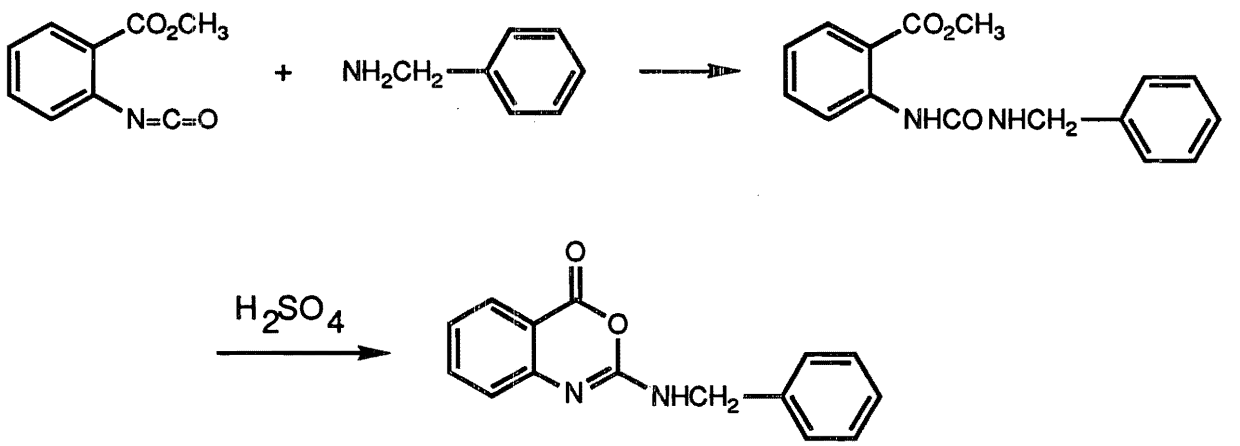
Progress Report. Three new compounds have been synthesized this quarter and submitted for testing as antivesicants. The compounds are 2-phenylamino-4H-3,1-benzoxazin-4-one, 2-Benzylamino-4H-3,1-benzoxazin-4-one, and 4-cyanophenyl p-guanidino benzoate. The structures are shown below.



2-Phenylamino benzoxazin-4-one was prepared by the cyclodehydration of 2-(3-phenylureido)benzoic acid. The latter was prepared by the reaction of phenyl isocyanate with anthranilic acid in THF.



2-Benzylamino-4H-3,1-benzoxazin-4-one was also prepared by a cyclodehydration reaction using H_2SO_4 as the dehydrating agent. The starting compound for this reaction was methyl 2-(3-benzylureido)benzoate. This in turn was prepared by the reaction of 2-carbomethoxy phenylisocyanate with benzyl amine in THF at room temperature.



4-Cyanophenyl *p*-guanidinobenzoate was prepared from *p*-guanidinobenzoic acid and 4-cyanophenol using DCC as the coupling agent.

Biological Assays. The new compounds have not yet been assayed with various proteases

EXPERIMENTAL SECTION

2-Phenylamino-4H-3,1-benzoxazin-4-one. Phenyl isocyanate (3.57 g, 0.03 mol) was dissolved in 10 mL THF and to this stirred solution was added anthranilic acid (4.1 g, 0.03 mol) and the reaction stirred overnight at room temperature. The solvent was then removed under reduced pressure to get 2-(3-phenylureido) benzoic acid as a white solid.

The product from the above reaction (3.84 g) was dissolved in 10 mL conc. H_2SO_4 and kept at room temperature for 1 h. The reaction was then poured over ice, neutralized with saturated $NaHCO_3$ solution, the precipitated white solid filtered and dried in air and recrystallized from benzene to get the title compound as white crystals; m.p. 191-192 °C. ^1H NMR (DMSO-d_6) δ: 7.1-8.0 (aromatic). Anal. Calcd.for $C_{14}H_{10}N_2O_2$ 0.27 H_2O : C, 69.13; H, 4.12; N, 11.52. Found: C, 69.10; H, 4.32; N, 10.71.

2-Benzylamino-4H-3,1-benzoxazin-4-one. To a stirred solution of benzylamine (634 mg, 0.0059 mol) in dry THF (40 mL) was added 2-carbomethoxy phenylisocyanate (1 g, 0.0056 mol) and the reaction stirred at room temperature overnight. The solvent was removed under reduced pressure to obtain methyl 2-(3-benzylureido) benzoate as a white solid.

The product (1.13 g) from the reaction above was dissolved in 2 mL conc. H_2SO_4 and left at room temperature for 1 h. The reaction was worked up as in the previous experiment and the product recrystallized from ethyl acetate-benzene to get the title compound as a white solid; m.p. 177-178 °C. ^1H NMR (DMSO-d_6) δ: 4.3 (d, 2H, $-CH_2-$), 6.9-8.4 (aromatic), 10.2 (s, 1H, NH). Anal. Calcd. for $C_{15}H_{12}N_2O_2$ x 1.1 H_2O: C, 66.17; H, 5.22; N, 10.29. Found: C, 66.20; H, 5.24; N, 10.15.

4-Cyanophenyl *p*-Guanidino Benzoate. Dicyclohexyl carbodiimide (2.33 g, 11.3 mmol) was added to a solution of *p*-guanidinobenzoic acid hydrochloride (2.43 g, 11.3 mmol) in 50 mL pyridine. 4-Cyanophenol (1.34 g, 11.3 mmol) was added to this

solution and the resulting mixture was stirred at room temperature for 14 h with protection from moisture. Dicyclohexylurea was removed from the reaction mixture by filtration and the filtrate was concentrated *in vacuo* to a foam which was triturated with 30 mL EtOAc. The beige solid that resulted was collected by filtration and washed throughly with EtOAc. The crude product (2.5 g) is taken up in approx. 3 L of water and the solid remaining is removed by filtration through a bed of Celite. The filtrate was lyophilized to obtain 1.75 g (70 %) of pure product; m.p. 160-62 °C. ^{1}H NMR (d_6-Me_2SO) δ: 10.58 (s, 1H), 8.18 (d, 2H), 7.99 (d, 2H), 7.88 (br.s., 3H), 7.52 (d, 2H), 7.42 (d, 2H). Anal. Calcd. for $C_{15}H_{13}ClN_4O_2$ x 1.98 H_2O: C, 51.13; H, 4.85; N, 15.90. Found: C, 51.13; H, 4.70; N, 15.79.

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #7

Reporting Period: August 1, 1990 to October 31, 1990

Report Date: November 12, 1990

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

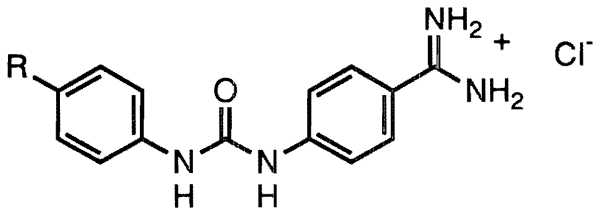
Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Girish S. Patil	Research Scientist	100%

Approximate Contract Expenditures to October 31, 1990

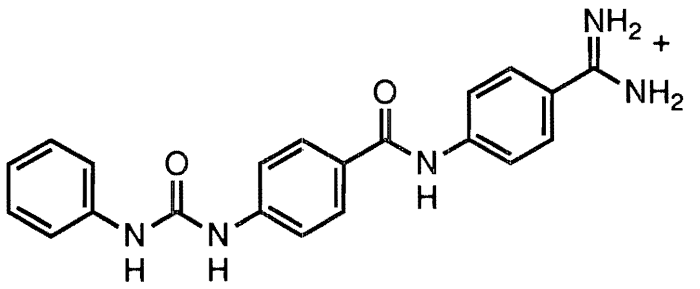
Personnel	$112,315
Fringe	23,074
Supplies	17,231
Travel	2,071
Equipment	595
Overhead	70,473
Total	$225,759

Progress Report. This quarter, we have focused on the synthesis of inhibitors for tryptase and related enzymes such as plasminogen activator. Three new compounds have been synthesized this quarter and one was submitted for testing as an antivesicant. The other two will be submitted shortly. The compounds are 1-(4-amidinophenyl)-3-phenylurea • hydrochloride (**1**), 1-(4-amidinophenyl)-3-(4-chlorophenyl)urea • hydrochloride (**2**), and N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide (**3**). The structures are shown below.



1 R = H

2 R = Cl

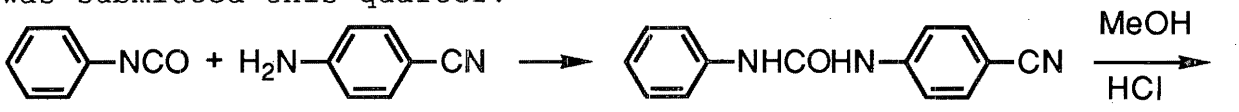


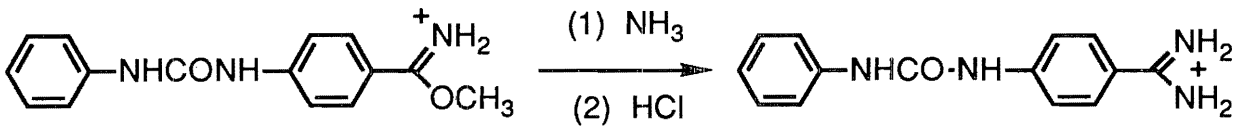
3

The inhibitory potency of these compounds and several compounds which we submitted in the previous quarters was tested towards porcine pancreatic elastase (PPE), human leukocyte elastase (HLE), chymotrypsin, cathepsin G, trypsin and human lung tryptase. The values for the inhibition constants are reported in the table in the Biological Assays section.

Synthesis. The synthesis of *p*-guanidino benzoic acid derivatives was discontinued due to numerous problems in the synthetic/purification procedures when the substituents on the carboxyl group were more complex aromatic structures than a single phenyl group. We are presently concentrating on derivatives of 4-aminobenzamidine as inhibitors for trypsin-like enzymes. Although the synthesis and purification procedures are still difficult, better results are being obtained with more complex aromatic structures containing benzamidine-like moieties.

Numerous attempts to make 1-(4-amidinophenyl)-3-phenylurea (**1**) and its 4-chloro analog (**2**) by condensing 4-aminobenzamidine with phenylisocyanate and 4-chlorophenylisocyanate failed. These compounds were finally made by a different route. 1-(4-Amidinophenyl)-3-phenylurea (**1**) was finally prepared starting from 4-aminobenzonitrile. Phenylisocyanate and 4-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by treatment with dry methanol in the presence of dry HCl. The final amidino compound was obtained by refluxing the imidate ester in isopropanol saturated with dry ammonia. The product was isolated as its hydrochloride salt and was submitted this quarter.

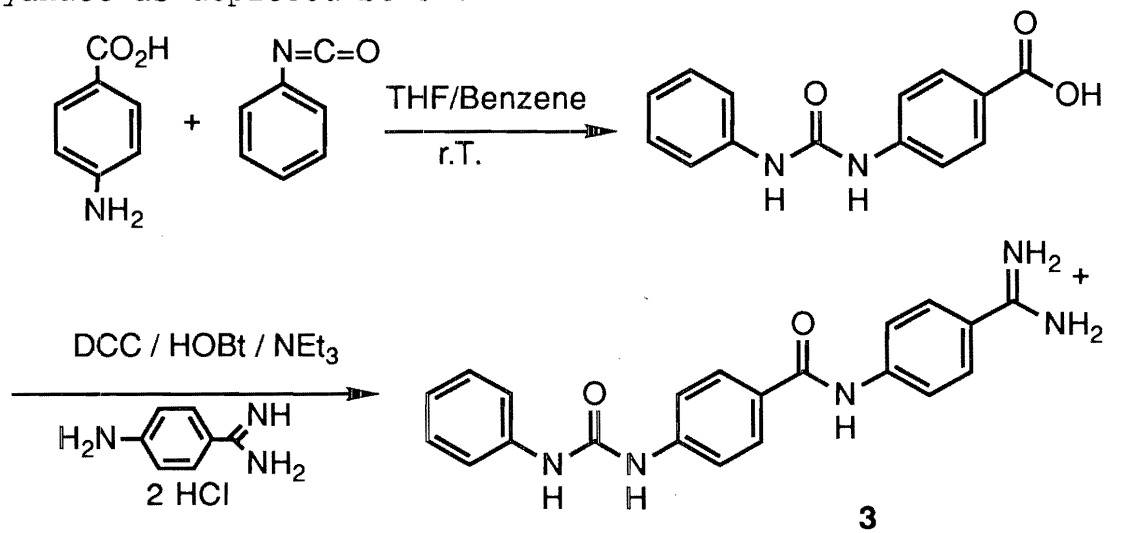




Submitted

1-(4-Amidinophenyl)-3-(4-chlorophenyl)urea (**2**) was prepared using the same reaction scheme except that phenylisocyanate was replaced by 4-chlorophenylisocyanate. This compound is ready to be submitted.

N-(4-Amidinophenyl)-4-(phenylcarbamoylamino)benzamide (**3**) was synthesized by DCC coupling of 4-(phenylcarbamoylamino)benzoic acid with 4-aminobenzamidine using 1-hydroxybenzotriazole in DMF. The acid was prepared from 4-aminobenzoic acid and phenyl isocyanate as depicted below.



3
Ready to Submit

An attempted synthesis of this compound by the acid chloride method gave very complex reaction mixtures from which no product could be isolated. In addition, the desired product was not formed when 1-hydroxybenzotriazole was omitted in the DCC coupling procedure and instead we obtained the N-acyl urea derivative of DCC and the starting acid. N-Acyl ureas are isolated as byproducts of DCC coupling reactions when the intermediate O-acyl urea fails to react fast enough with the amine component in the reaction mixture.

Biological Assays. Two of the inhibitors showed moderate inhibition of porcine pancreatic elastase and chymotrypsin and two were excellent trypsin and human lung tryptase inhibitors. These lead compounds will be pursued to prepare yet better inhibitors. Results are summarized in Table I.

EXPERIMENTAL SECTION

1-(4-Amidinophenyl)-3-phenylurea. To a stirred solution of 4-aminobenzonitrile (4.72 g, 0.04 mol) in dry benzene (200 mL) was added phenylisocyanate (9.52 g, 0.08 mol) and the resulting solution refluxed for 5 h and further stirred at room temperature overnight. The precipitated solid was filtered and recrystallized from methanol to get the urea as a white crystalline solid (8.3 g).

Dry HCl gas was passed through a solution of the urea (4.74 g, 0.02 mol) in dry dimethoxyethane (100 mL) for 1 h. The reaction mixture was then stirred at room temperature for 24 h. The precipitated imidate ester was filtered and stored over KOH in a vacuum desiccator (3.5 g) for 24 h.

The imidate ester (2 g, 0.006 mol) was dissolved in isopropanol saturated with ammonia (100 mL) and the reaction mixture was refluxed for 6 h and further stirred at room temperature overnight. The solvent was then evaporated under reduced pressure, a white solid that was obtained, and this was recrystallized from 2N HCl to get the title compound as its HCl salt (1.5 g); m. p. 240 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 7.00 (t, 1H), 7.30 (t, 2H), 7.45 (d, 2H), 7.65 (d, 2H), 7.80 (d, 2H), 8.80 (s, 2H), 9.15 (s, 2H), 9.40 (bs, 1H), 9.80 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}ClN_4O \cdot H_2O$: C, 54.53; H, 5.56; Cl, 11.35; N, 18.18. Found: C, 54.53; H, 5.46; Cl, 11.42; N, 18.13.

Table I. Inhibition of Bovine Trypsin, Human Lung Tryptase, Human Leukocyte Elastase, Porcine Pancreatic Elastase, Chymotrypsin, and Cathepsin G by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Benzoxazinones and Aminobenzamidine Derivatives.[a]

	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)					
Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	HLE[d]	PPE[d]	ChT[d]	CatG[d]
Isocoumarin Derivatives						
4-chloro-3-methyl IC	NT[e]	NT[e]	72%[f]	NI[g]	66	8
4-chloro-3-methoxy IC	NT[e]	NT[e]	87	601	206	25
Benzoxazinones						
2-phenylamino-4H-3,1-benzoxazin-4-one	NT[e]	NT[e]	NI[g]	216	77	reactivates
2-benzylamino-4H-3,1-benzoxazin-4-one	NT[e]	NT[e]	NI[g]	NI[g]	NI[g]	NI[g]
Guanidinobenzoic Acid Derivatives						
4-cyanophenyl *p*-guanidinobenzoate	150,000	91,000	NT[e]	NT[e]	NT[e]	NT[e]
Aminobenzamidine Derivatives						
N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide	14,000	600	NT[e]	NT[e]	NT[e]	NT[e]

Table III (Continued).

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[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; Human Lung Tryptase, 0.02 μM; HLE, 0.3 μM; PPE, 1.6 μM; Chymotrypsin, 1.6 μM; Cathepsin G, 1.6 μM. Bovine trypsin and human lung tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM). HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM). PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM). Chymotrypsin (ChT) and Cathepsin G (CatG) were assayed with Suc-Val-Pro-Phe-NA (0.5 μM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-cyanophenyl *p*-guanidinobenzoate, 0.46 μM; N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide, 1.22 μM.

[c]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-cyanophenyl *p*-guanidinobenzoate, 0.22 μM; N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide, 11.6 μM.

[d]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were in the range 41 μM- 1mM.

[e]Not tested.

[f]Inhibition was not time dependent.

[g]No inhibition.

G-33-6

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #8

Reporting Period: November 1, 1990 to January 31, 1991

Report Date: February 8, 1991

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

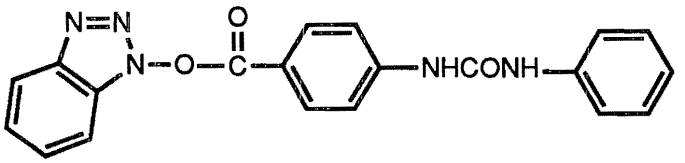
James C. Powers	PI	25%
Maria Hernandez	Research Scientist	100%
Girish S. Patil	Research Scientist	100%

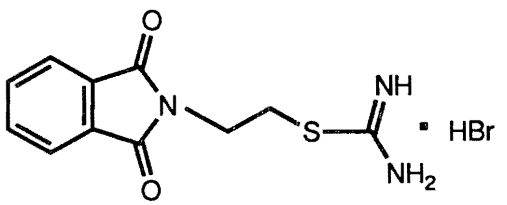
The percent time for the next quarter will change. Dr. Hernandez left this project at the end of the quarter and will not be reduced.

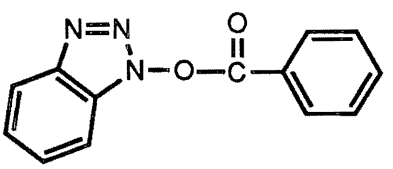
Approximate Contract Expenditures to December 31, 1990

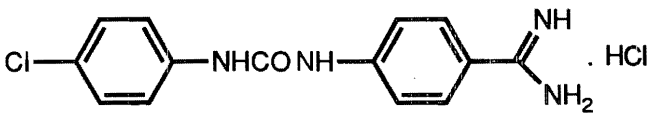
Personnel	$122,568
Fringe	25,770
Supplies	22,081
Travel	2,443
Equipment	595
Overhead	107,670
Total	$281,127

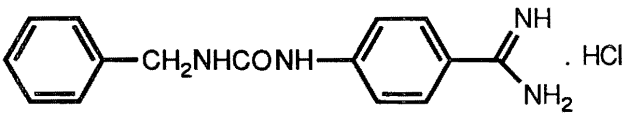
Progress Report. This quarter we have continued the synthesis of inhibitors for the tryptase family of serine proteases. Six new compounds were synthesized this quarter and submitted for testing as antivesicants. The compounds are 1,2,3-benzotriazole-1-(4-phenylcarbamoylaminophenyl)carboxylate, N-(2-isothioureido)ethyl phtalimide hydrobromide, 1,2,3-benzotriazole-1-benzoate, 1-(4-amidinophenyl)-3-(4-chlorophenyl)urea, 1-(4-amidinophenyl)-3-(4-chlorophenyl)urea, and 1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea. Their structures are shown below.











The inhibitory potency of these compounds was tested towards trypsin. A complete compilation of the biological data obtained with the compounds submitted to date will be included in the Annual Report.

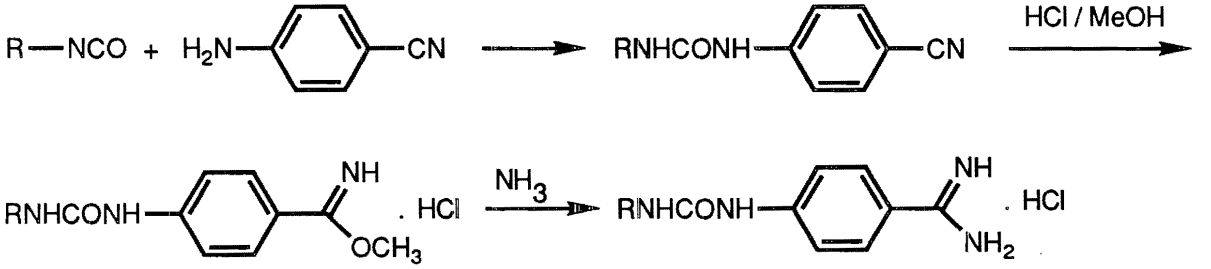
Synthesis. The synthesis of N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide was discussed in the last quarterly report and at that time the compound was reported as a mixture of salts because the elemental analysis could not be matched with the expected structure. The fact that it was a good trypsin and human lung tryptase inhibitor further suggested that it must contain the amidino moiety in its structure. On the other hand, all the analytical data could be explained by a compound with the structure corresponding to an acylated 1-hydroxy-1,2,3-benzotriazole, namely, 1,2,3-benzotriazole-1-(4-phenylcarbamoyl aminophenyl)carboxylate. The compound is therefore reported as such in the Experimental Section. A spectrophotometric study of the hydrolysis of this compound in the buffer used to assay inhibition of trypsin shows at least two isosbestic points over a 5 h period. However, no significant hydrolysis was observed during the short time periods used in the inhibition assays and therefore the inhibition constants reported are accurate values. Kinetic studies are being performed to elucidate a mechanism for the inhibition of trypsin by this compound. The benzoyl derivative was made in order to compare its inhibition of trypsin with that of the more complex derivative discussed above. It was also made in order to ascertain if an acyl derivative of 1-hydroxy-1,2,3-benzotriazole could indeed inhibit trypsin. We

found this to be the case, and 1,2,3-benzotriazole-1-benzoate had a $k_{obs}/[I]$ value of 1,100 $M^{-1}s^{-1}$ towards trypsin. It was made by acylation of 1-hydroxy-1,2,3-benzotriazole with benzoyl chloride in THF/pyridine. This type of inhibitors are a new and interesting class of trypsin inhibitors and mechanistic studies will help us elucidate their mode of action.

The synthesis of N-(2-isothioureido)ethyl phtalimide hydrobromide was done by alkylation of thiourea with N-(2-bromoethyl)phtalimide in THF. This compound was modeled after the isothioureido isocoumarins prepared in our laboratories which have showed significant inhibitory potency towards trypsin and trypsin-like enzymes.

1-(4-Amidinophenyl)-3-(4-chlorophenyl)urea, 1-(4-amidinophenyl)-3-benzylurea and 1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea were made using essentially the same set of reactions. Some modifications were required in going from one product to the other. These changes are clearly noted in the Experimental Section. Following is a general procedure used for making all the amidino compounds submitted so far.

4-Aminobenzonitrile was condensed with the corresponding isocyanate in refluxing benzene. The resulting urea was converted to the imidate ester by treatment with methanol in the presence of HCl. The imidate ester on refluxing with isopropanol saturated with dry ammonia gave the desired amidino compound. All the amidino compounds were isolated as their hydrochloride salts. A general reaction scheme is shown below.



EXPERIMENTAL SECTION

1,2,3-Benzotriazole-1-(4-phenylcarbamoylaminophenyl) carboxylate. Triethylamine (0.19 mL, 1.4 mmol) was added to a solution of 4-aminobenzamidine dihydrochloride (284 mg, 1.4 mmol) in 6 mL DMF. 1,3-Dicyclohexylcarbodiimide (311 mg, 1.5 mmol) was then added, followed by 1-hydroxy-1,2,3-benzotriazole (185 mg, 1.4 mmol). This mixture was cooled to 10 °C and 4-phenylaminocarbamoyl benzoic acid was then added (350 mg, 1.4 mmol). The reaction mixture was stirred at 5-10 °C for 14 h and dicyclohexylurea was removed by filtration. It was washed with fresh DMF, and the filtrate was concentrated to dryness and coevaporated with acetone to obtain an amorphous white solid that was washed with $CHCl_3$:EtOH 4:1 to obtain 225 mg (40 %) of pure product as a white solid; mp 217-8 °C (dec). The product can be recrystallized from $CHCl_3$/DMF/hexane. ^1H NMR (DMSO-d_6) δ: 9.45 (br s, 1H); 8.96 (br s, 1H); 8.22 (d, 2H); 8.18 (d, 1H); 7.85 (d, 1H); 7.78 (d, 2H); 7.67 (t, 1H); 7.54 (t, 1H); 7.49 (d, 2H); 7.32 (t, 2H); 7.02 (t, 1H). Anal Calcd. for $C_{20}H_{15}N_5O_3 \cdot 0.2\ H_2O$: C, 63.71; H, 4.12; N, 18.58. Found: C, 63.71; H, 4.36; N, 18.80.

N-(2-Isothioureido)ethyl phtalimide hydrobromide. N-(2-Bromoethyl)phtalimide (4.6 g, 18 mmol) was dissolved in 50 mL THF and then thiourea (1.52 g, 20 mmol) was added in one portion. The resulting mixture was heated to reflux temperature for 2 days and then allowed to cool to 5-10 °C in an ice bath. The white solid in suspension was filtered and washed with THF and hexane to yield 3.21 g (54%) of pure product; mp 245-6 °C (dec). ^1H NMR (DMSO-d_6) δ: 9.04 (br s, 4H); 7.86-7.91 (m, 4H); 3.87 (t, 2H); 3.49 (t, 2H). Anal. Calcd. for $C_{11}H_{12}BrN_3O_2S$: C, 40.01; H, 3.66; N, 12.73; Br, 24.20. Found: C, 40.10; H, 3.71; N, 12.72; Br, 24.14.

1,2,3-Benzotriazole-1-benzoate. A solution of benzoyl chloride (6.2 mL, 53 mmol) in 30 mL of THF was added dropwise to a

pyridine (4.3 mL, 53 mmol) in 65 mL THF. The resulting mixture was stirred at room temperature for 24 h under protection from moisture, and the solvent was then removed under vacuum. The white solid obtained was purified by low temperature recrystallization from $CHCl_3$/hexane and cooling at 0-5 °C for 2 days. The pure product was obtained as a white solid (5.15 g, 47 %); mp 78-9 °C. A second crop can be obtained from the mother liquor by concentration to 2/3 of the volume, addition of hexane, and further cooling at 0-5 °C for 2 days. ^1H NMR (DMSO-d_6) δ: 7.98-7.92 (m, 3H); 7.72 (d, 1H); 7.64-7.38 (m, 5H). Anal. Calcd. for $C_{13}H_9N_3O_2$: C, 65.26; H, 3.79; N, 17.56. Found: C, 65.36; H, 3.81; N, 17.61.

1-(4-Amidinophenyl)-3-(4-chlorophenyl)urea hydrochloride. To a stirred solution of 4-aminobenzonitrile (4.72 g, 0.04 mol) in dry THF (50 mL) was added 4-chlorophenylisocyanate (6.12 g, 0.04 mol) in one portion and the reaction stirred magnetically overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (9.2 g, 85 %).

Dry HCl was passed through a cooled solution of the cyano urea (5.43 g, 0.02 mol) and dry methanol (3.2 g, 0.10 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction further stirred at room temperature for 36 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained as a yellow solid was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (6 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (4.8 g, 84 %); mp 275 °C (dec.). ^1H NMR (DMSO-d_6) δ: 7.32 (d, 2H); 7.50 (d, 2H); 7.66 (d, 2H); 7.80 (d, 2H); 8.85 (s, 2H); 9.18 (s, 2H); 9.70 (s, 1H); 9.98 (s, 1H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O \cdot 0.69\ H_2O$: C, 49.80; H,

4.58; Cl, 21.01; N, 16.59. Found: C, 50.21; H, 4.62; Cl, 20.73; N, 16.19.

1-(4-Amidinophenyl)-3-benzylurea hydrochloride. To a stirred solution of 4-aminobenzonitrile (3.9 g, 0.03 mol) in dry benzene (100 mL) was added benzylisocyanate (4.5 g, 0.03 mol) in one portion and the reaction heated under reflux for 6 h and stirred magnetically at room temperature overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (6.1 g, 72 %).

Dry HCl was passed through a cooled solution of the cyano urea (3 g, 0.012 mol) and dry methanol (1.92 g, 0.060 mol) in dry dimethoxyethane (150 mL) for 2 h and the reaction further stirred at room temperature for 24 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained as a yellow solid was stored in a vacuum desiccator over KOH for 24 h (3.2 g).

The imidate ester (3.2 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained recrystallized from 2N HCl to get white crystals of the title compound (3 g, 84 %); mp 292 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 4.31 (d, 2H); 7.17 (t, 1H); 7.20-7.38 (m, 5H); 8.82 (s, 2H); 9.12 (s, 2H); 9.66 (s, 1H). Anal. Calcd. for $C_{15}H_{17}Cl_2N_4O$: C, 59.11; H, 5.62; Cl, 11.63; N, 18.38. Found: C, 59.23; H, 5.64; Cl, 11.63; N, 18.28.

1-(4-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride. To a stirred solution of 4-aminobenzonitrile (2.95 g, 0.025 mol) in dry benzene (50 mL) was added 4-phenoxyphenylisocyanate (5.25 g, 0.025 mol) in one portion and the reaction heated under reflux for 10 h and stirred at room temperature overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (6.5 g, 79 %).

Dry HCl was passed through a cooled solution of the cyano urea (5 g, 0.015 mol) and dry methanol (2.43 g, 0.075 mol) in dry

dimethoxyethane (100 mL) for 2 h and the reaction stored in the refrigerator for 48 h. Solvent was then evaporated under reduced pressure and the solid obtained stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (5 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 7 h and further stirred at room temperature for 24 h. Solvent was evaporated and the solid obtained recrystallized from 2N HCl to get white crystals of the title compound (4.6 g, 95 %); mp 202-205 °C. ^{1}H NMR (DMSO-d_6) δ: 6.90-7.01 (m, 4H); 7.09 (t, 1H); 7.35 (m, 2H); 7.47 (m, 2H); 7.65(d, 2H); 7.80 (d, 2H); 8.80 (s, 2H); 9.15 (s, 2H); 9.39 (s, 1H); 9.75 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.7\ H_2O$: C, 60.74; H, 5.16; Cl, 8.98; N, 14.17. Found: C, 60.36; H, 5.05; Cl, 9.00; N, 14.08.

Q-33650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #9

Reporting Period: February 1, 1991 to April 30, 1991

Report Date: May 13, 1991

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

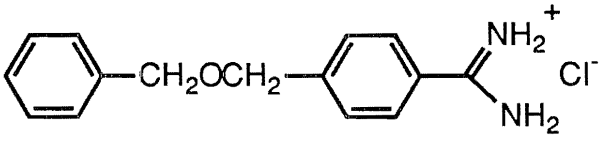
Current Staff and Percent Time on the Project

James C. Powers	PI	25%
Girish S. Patil	Research Scientist	100%

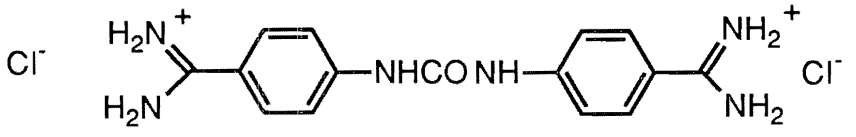
Approximate Contract Expenditures to April 30, 1991

Personnel	$130,734
Fringe	27,917
Supplies	26405
Travel	2485
Equipment	595
Overhead	116,845
Total	$304,981

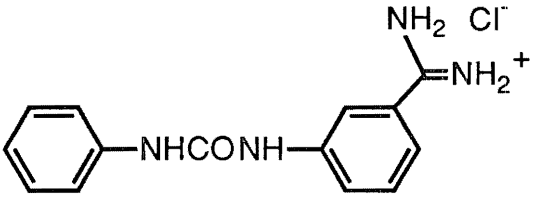
Progress Report. We continued to work on the synthesis of inhibitors for trypsin-like enzymes this quarter. Five new compounds were synthesized and submitted for testing as antivesicants this quarter. The compounds are (4-amidinobenzyl) benzyl ether hydrochloride (**GP 9**), bis(4-amidinophenyl)urea dihydrochloride (**GP 10**), 1-(3-amidinophenyl)-3-phenylurea hydrochloride (**GP 11**), (4-amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**), 1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**). Their structures are shown below.



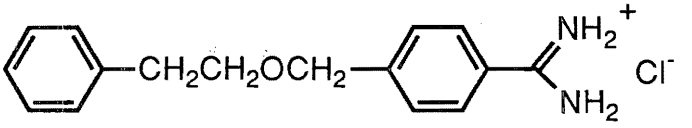
GP 9



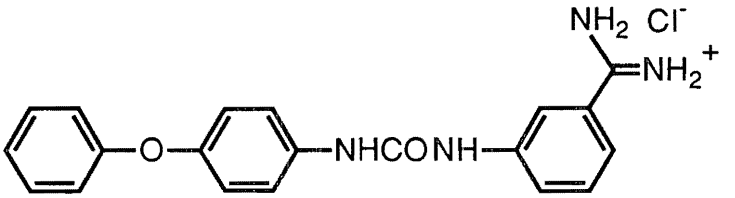
GP 10



GP 11



GP 12

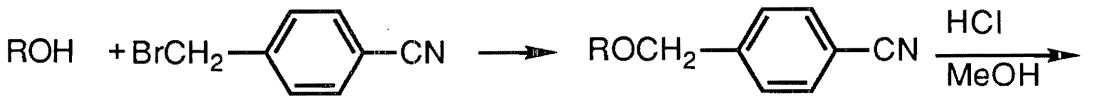


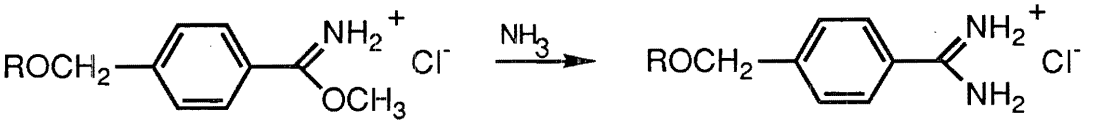
GP 13

The inhibitory potency of these compounds was tested with trypsin. A complete compilation of the biological data obtained for the compounds submitted to date will be included in the Final Report.

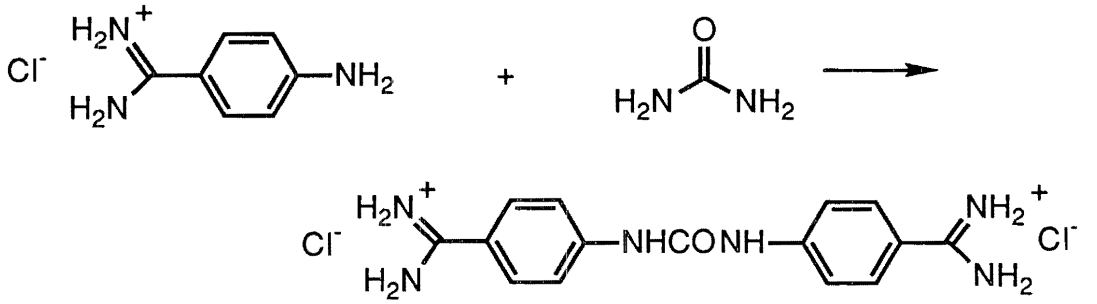
Synthesis. (4-Amidinobenzyl)benzyl ether (**GP 9**) was synthesized from the cyano ether obtained by the reaction of sodium benzyloxide and α-bromo-*p*-tolunitrile. The cyano ether was converted to the imidate ester by treating it with dry methanol in the presence of HCl. The imidate ester on reacting with ammonia gave the desired amidino compound.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**) was prepared by replacing benzyl alcohol with phenylethyl alcohol and keeping the rest of the procedure as in **GP 9**.



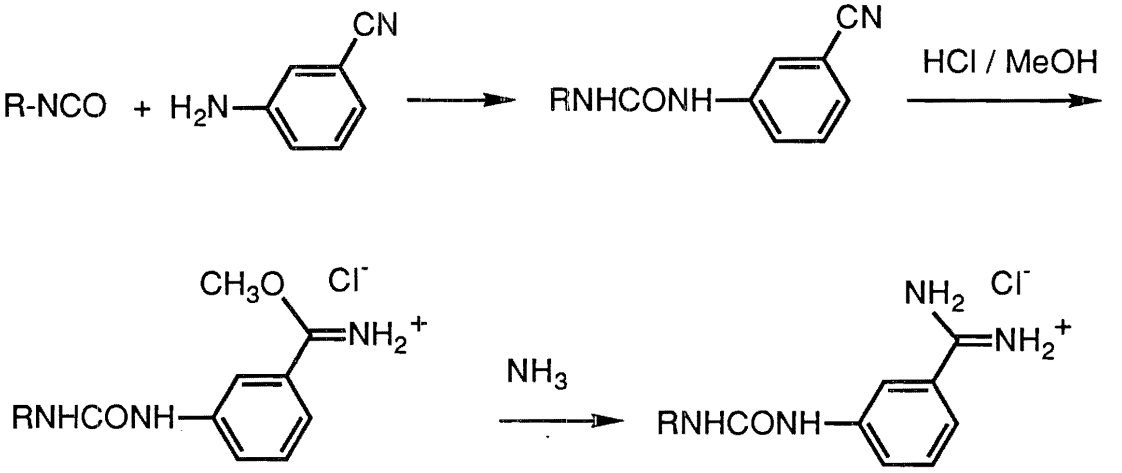


Bis(4-amidinophenyl)urea dihydrochloride (**GP 10**) was made by condensing 4-aminobenzamidine dihydrochloride with urea.



1-(3-amidinophenyl)-3-phenylurea hydrochloride (**GP 11**) was prepared starting from 3-aminobenzonitrile. Phenyl isocyanate and 3-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by the treatment with dry methanol in presence of dry HCl. Refluxing the imidate ester in dry isopropanol saturated with ammonia afforded the desired amidino compound.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**) was prepared using the procedure described above except that phenyl isocyanate was replaced with 4-phenoxyphenyl isocyanate.



EXPERIMENTAL SECTION

(4-Amidinobenzyl)benzyl ether hydrochloride (GP 9). A solution of benzyl alcohol (1.08 g, 0.01 mol) in dry THF (10 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (1.96 g, 0.01 mol) in dry THF (10 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.41 g, 44 %); mp 102-104 °C. ^{1}H NMR (DMSO-d_6) δ: 4.58 (s, 2H); 4.65 (s, 2H); 7.25-7.40 (m, 5H); 7.59 (d, 2H); 7.84 (d, 2H); 9.23 (bs, 2H); 9.41 (bs, 2H). Anal. Calcd. for $C_{15}H_{17}Cl_1N_2O$: C, 65.10; H, 6.19 Cl, 12.81; N, 10.12. Found: C, 64.98; H, 6.20; Cl, 12.73; N, 10.02.

Bis(4-amidinophenyl)urea dihydrochloride (GP 10). A suspension of urea (0.60 g, 0.01 mol) and 4-aminobenzamidine (4.16 g, 0.02 mol) in water (5 mL) was heated under reflux for 24 h. The condenser was then removed and the heating further continued for 24 h. The reaction was then trichurated with water (25 mL). The solid was filtered out dissolved in water and the clear solution acidified with HCl to get the product as a white solid

(3.1 g, 84 %); mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 7.68 (d, 4H); 7.84 (d, 4H); 9.00 (s, 4H); 9.23 (d, 4H); 10.45(s, 2H). Anal. Calcd. for $C_{15}H_{18}Cl_2N_6O \cdot 0.3\ H_2O$: C, 48.07; H, 4.96; Cl, 18.96; N, 22.43. Found: C, 48.06; H, 5.05; Cl, 19.03; N, 22.55.

1-(3-Amidinophenyl)-3-phenylurea hydrochloride (GP 11). To a stirred solution of 3-aminobenzonitrile (4.72 g, 0.04 mol) in benzene (100 mL) was added phenyl isocyanate (5.00 g, 0.042 mol) and the reaction refluxed for 5 h and further stirred at room temperature overnight. The separated white solid was filtered out and recrystallised from methanol (8.2 g, 84 %).

Dry HCl was passed through a cooled solution of the cyano urea (4.00 g, 0.016 mol) and dry methanol (2.7 g, 0.08 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction stored in a refrigerator for 14 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained, as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (4 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white solid of the title compound (2.1 g, 55 %); mp 252-254 °C. ^{1}H NMR (DMSO-d_6) δ: 6.98 (t, 1H); 7.20-7.35 (m, 3H); 7.40-7.55 (m, 3H); 7.70 (d, 1H); 7.95 (s, 1H); 9.1 (bs, 2H); 9.32 (bs, 2H); 9.45 (bs, 1H); 9.18 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}Cl_1N_4O$: C, 57.83; H, 5.20 Cl, 12.19; N, 19.27. Found: C, 57.74; H, 5.20; Cl, 12.16; N, 19.21.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (GP 12). This compound was prepared using the procedure described for compound **GP 9** and replacing benzyl alcohol with phenyl ethyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 2.88 (t, 2H); 3.69 (t, 2H); 4.60 (s, 2H); 7.15-7.30 (m, 5H); 7.49 (d, 2H); 7.80 (d, 2H); 9.17 (bs, 2H); 9.37 (bs, 2H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O$: C, 66.09; H, 6.59; Cl, 12.19; N, 9.63. Found: C, 66.18; H, 6.61; Cl, 12.09; N, 9.56.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (GP 13). This compound was prepared using the

procedure described for compound **GP 11** and substituting 4-phenoxyphenyl isocyanate for phenyl isocyanate. ^{1}H NMR (DMSO-d_6) δ: 6.91-7.20 (m, 4H); 7.10 (t, 1H); 7.30-7.40 (m, 3H); 7.45-7.58 (m, 3H); 7.70(d, 1H); 7.95 (s, 1H); 9.00 (s, 2H); 9.34 (s, 1H); 9.36 (s, 2H); 9.55 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.75$ H_2O: C, 60.60; H, 5.17; Cl, 8.96; N, 14.14. Found: C, 60.62; H, 5.19; Cl, 8.96; N, 14.05.

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Quarterly Report #10

Reporting Period: May 1, 1991 to July 31, 1991

Report Date: August 25, 1991

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

(404) 894-4038

Current Staff and Percent Time on the Project

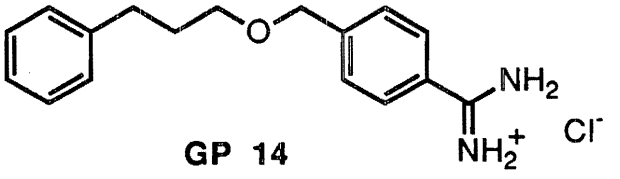
James C. Powers	PI	25%
Girish S. Patil	Research Scientist	100%

Dr. Patil worked on this project until the end of June and so the percent time represents only May and June. For the quarter, his percent time would be 66.7 %.

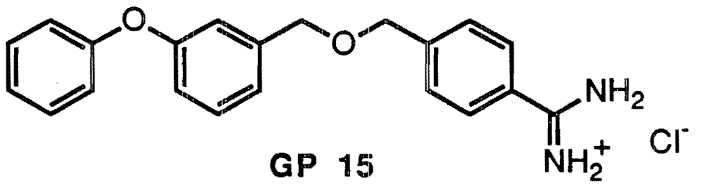
Approximate Contract Expenditures to July 31, 1991

Personnel	$133,901
Fringe	28,751
Supplies	27,831
Travel	2,515
Equipment	595
Overhead	120,128
Total	$313,721

Progress Report. In the final three months of the project work on the sythesis of tryptase inhibitors was continued. Two new compounds were synthesized and submitted for testing as antivesicants during this period. The compounds are (4-amidinobenzyl)-3-phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**). Their structures are shown below.



GP 14

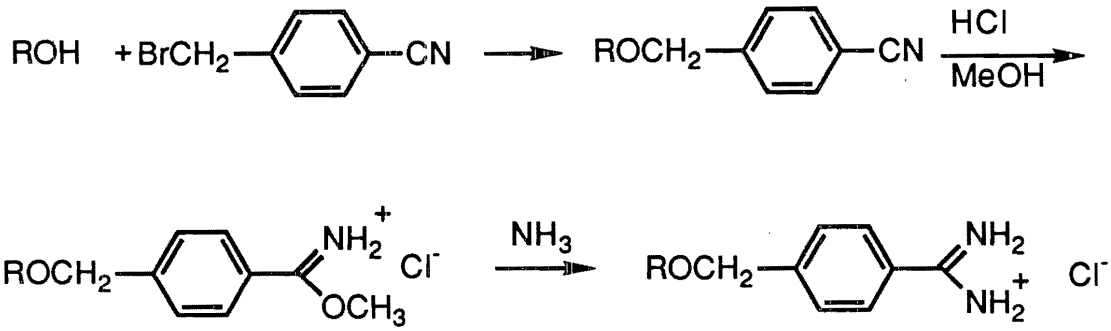


GP 15

A complete compilation of the biological data obtained for the compounds submitted to date will be included in the Final Report.

Synthesis. (4-Amidinobenzyl)-3-phenylpropyl ether (**GP 14**) was synthesized from the cyano ether obtained by reaction of sodium phenylpropyloxide and α-bromo-*p*-tolunitrile. The cyano ether was converted to the imidate ester by treating it with dry methanol in the presence of HCl. The imidate ester on refluxing with a saturated solution of ammonia in isopropanol gave the desired amidino compound.

(4-Amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**) was prepared by replacing phenylpropyl alcohol with 3-phenoxybenzyl alcohol and keeping the rest of the procedure as in **GP 14**.



EXPERIMENTAL SECTION

(4-Amidinobenzyl)-3-phenylpropyl ether hydrochloride (GP 14). A solution of 3-phenylpropyl alcohol (2.72 g, 0.02 mol) in dry THF (20 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (3.92 g, 0.02 mol) in dry THF (20 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. The solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.295 g, 31 %); mp 76-78 °C. ^{1}H NMR (DMSO-d_6) δ: 1.84 (m, 2H); 2.62 (t, 2H); 3.45 (t, 2H); 4.55 (s, 2H); 7.10-7.90 (aromatic, 9H); 9.20 (bs, 2H); 9.45 (bs, 2H). Anal. Calcd. for $C_{17}H_{21}Cl_1N_2O$: C, 66.99; H, 6.94 Cl, 11.63; N, 9.19. Found: C, 67.05; H, 6.95; Cl, 11.54; N, 9.20.

(4-Amidinobenzyl)-3-phenoxylbenzyl ether hydrochloride (GP 15). This compound was prepared using the procedure described for compound **GP 14** and replacing 3-phenylpropyl alcohol with 3-phenoxylbenzyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 4.55 (s, 2H); 4.63 (s, 2H); 6.95-7.80 (aromatic, 13H); 9.16 (bs, 2H); 9.40 (bs, 2H). Anal. Calcd. for $C_{21}H_{21}Cl_1N_2O_2$: C, 68.38; H, 5.74; Cl, 9.61; N, 7.59. Found: C, 68.20; H, 5.78; Cl, 9.48; N, 7.44.

G-33-650

Protease Inhibitors as Antivesicants

Contract No. DAMD17-89-C-9008

Georgia Tech Research Corporation

Semi-Annual Report

Reporting Period: February 1, 1991 to July 31, 1991

Report Date: August 30, 1991

James C. Powers

School of Chemistry and Biochemistry

Georgia Institute of Technology

Atlanta, GA 30332

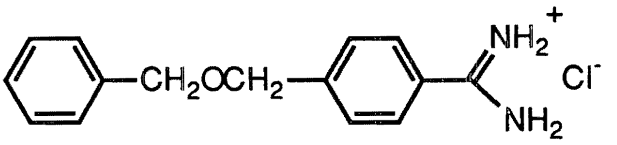
(404) 894-4038

PROGRESS REPORT

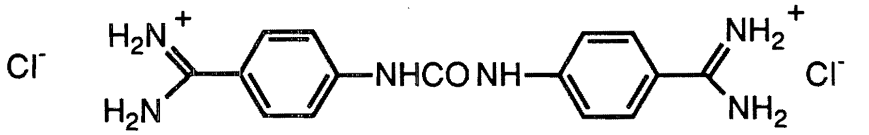
During the last portion of this contract, we focused on inhibitors for trypsin-like enzymes since Smith et al. [Smith, W. J., Cowan, F. M., and Broomfield, C. A. **1991**, FASEB Journal **5**, A828] have recently reported increased hydrolysis rates for substrates of trypsin-like enzymes (tryptases) by mustard stimulated lymphocytes. Thus, these enzymes may be significant proteases involved in blistering. Aromatic benzamidine derivatives are reversible inhibitors for trypsin-like enzymes and we prepared a series of derivatives to see if we could increase their inhibitory potency for tryptases. The most potent inhibitor thus far for both trypsin and the human lung tryptase is 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea (**GP 8**) which has a K_I value of 1.6 μM with bovine trypsin and inhibits 71% of the activity of human lung tryptase at 226 μM. Tables with complete biological data are included in the final report.

SYNTHESIS

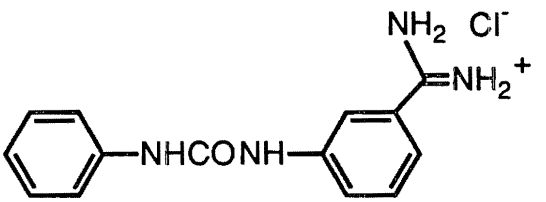
Benzamidines. Six new benzamidines were synthesized in the final six months of the project We successfully overcame the initial difficulties in the synthesis of these class of compounds and submitted a total of eleven benzamidines. The last compounds submitted are (4-amidinobenzyl) benzyl ether hydrochloride (**GP 9**), bis(4-amidinophenyl)urea dihydrochloride (**GP 10**), 1-(3-amidinophenyl)-3-phenylurea hydrochloride (**GP 11**), (4-amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**), 1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**), (4-amidinobenzyl)-3-phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**). The structures are shown below.



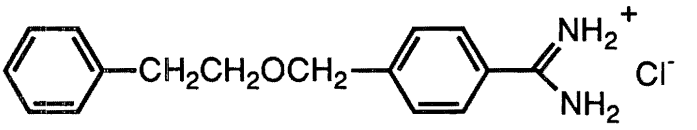
GP 9, BM08004



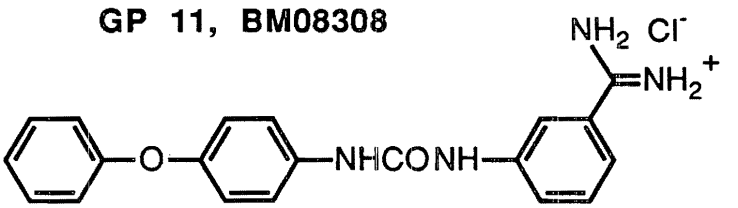
GP 10, BM08184



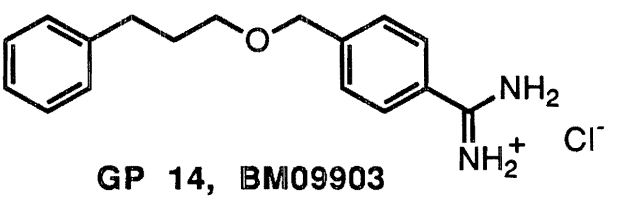
GP 11, BM08308



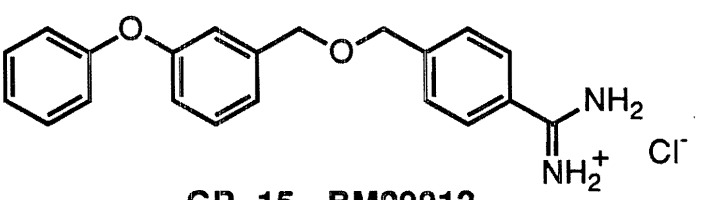
GP 12, BM08595



GP 13, BM08764



GP 14, BM09903

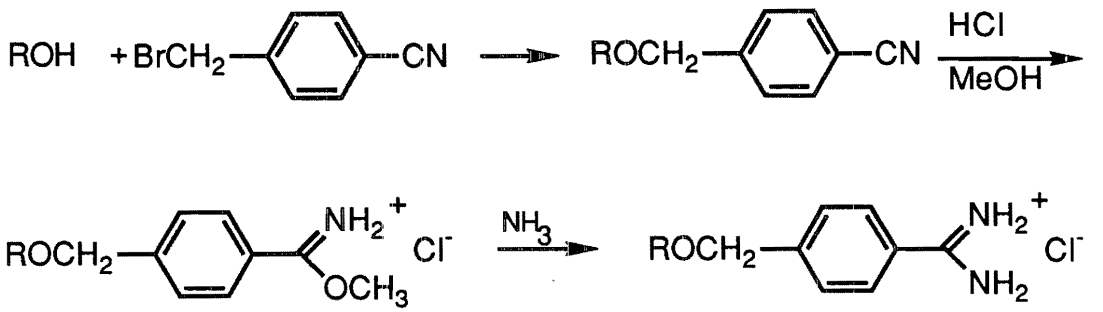


GP 15, BM09912

Synthetic Schemes. (4-Amidinobenzyl)benzyl ether (**GP 9**) was synthesized from the cyano ether obtained by the reaction of sodium benzyloxide and α-bromo-*p*-tolunitrile. The cyano ether was converted to the imidate ester by treating it with dry methanol in

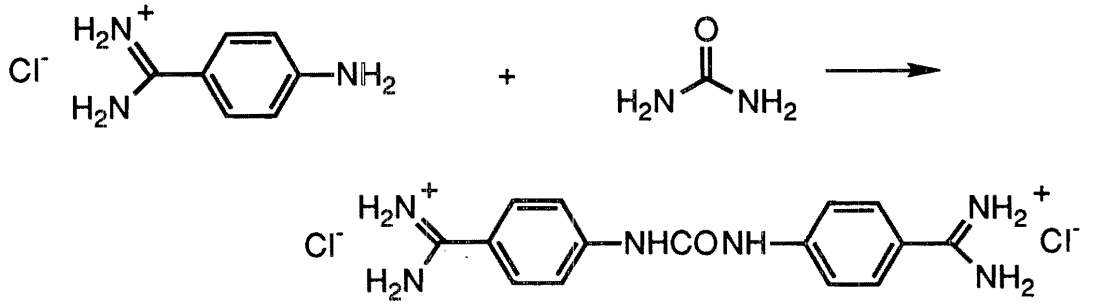
the presence of HCl. The imidate ester on reacting with ammonia gave the desired amidino compound.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**) was prepared by replacing benzyl alcohol with phenylethyl alcohol and keeping the rest of the procedure as in **GP 9**.



(4-Amidinobenzyl)phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**) were also synthesized using the same method with ROH being phenylpropyl alcohol and 3-phenoxybenzyl alcohol respectively.

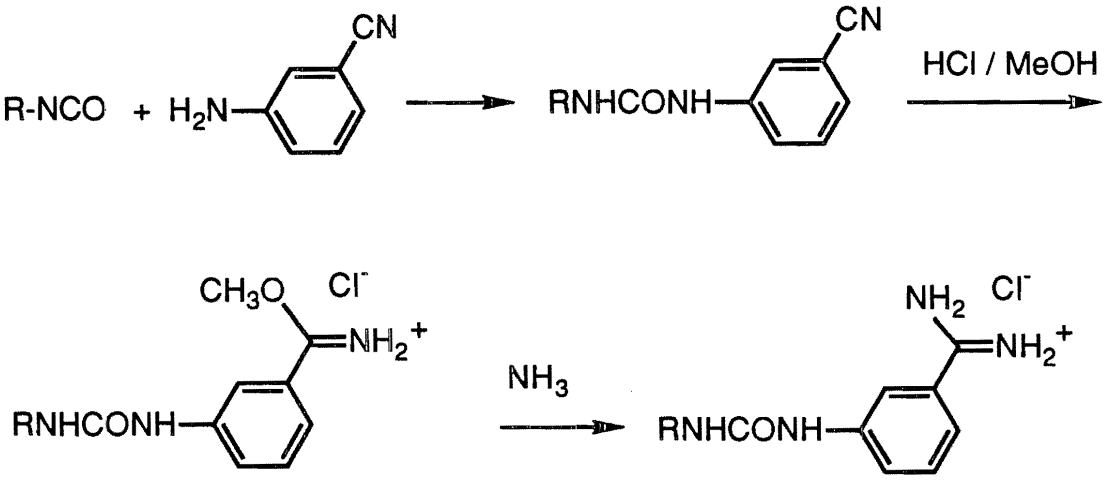
Bis(4-amidinophenyl)urea dihydrochloride (**GP 10**) was made by condensing 4-aminobenzamidine dihydrochloride with urea.



1-(3-Amidinophenyl)-3-phenylurea hydrochloride (**GP 11**) was prepared starting from 3-aminobenzonitrile. Phenyl isocyanate and 3-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by the treatment with dry methanol in presence of dry HCl. Refluxing the imidate

ester in dry isopropanol saturated with ammonia afforded the desired amidino compound.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**) was prepared using the procedure described above except that phenyl isocyanate was replaced with 4-phenoxyphenyl isocyanate.



(4-Amidinobenzyl)benzyl ether hydrochloride (GP 9). A solution of benzyl alcohol (1.08 g, 0.01 mol) in dry THF (10 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (1.96 g, 0.01 mol) in dry THF (10 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.41 g, 44 %); mp 102-104 °C. ^{1}H NMR (DMSO-d_6) δ: 4.58 (s, 2H); 4.65 (s, 2H); 7.25-7.40 (m, 5H); 7.59 (d, 2H); 7.84 (d, 2H); 9.23 (bs, 2H); 9.41 (bs, 2H). Anal. Calcd. for $C_{15}H_{17}Cl_1N_2O$: C, 65.10; H, 6.19 Cl, 12.81; N, 10.12. Found: C, 64.98; H, 6.20; Cl, 12.73; N, 10.02.

Bis(4-amidinophenyl)urea dihydrochloride (GP 10). A suspension of urea (0.60 g, 0.01 mol) and 4-aminobenzamidine (4.16 g, 0.02 mol) in water (5 mL) was heated under reflux for 24 h. The condenser was then removed and the heating further continued for 24 h. The reaction was then trichurated with water (25 mL). The solid was filtered out dissolved in water and the clear solution acidified with HCl to get the product as a white solid

(3.1 g, 84 %); mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 7.68 (d, 4H); 7.84 (d, 4H); 9.00 (s, 4H); 9.23 (d, 4H); 10.45(s, 2H). Anal. Calcd. for $C_{15}H_{18}Cl_2N_6O \cdot 0.3\ H_2O$: C, 48.07; H, 4.96; Cl, 18.96; N, 22.43. Found: C, 48.06; H, 5.05; Cl, 19.03; N, 22.55.

1-(3-Amidinophenyl)-3-phenylurea hydrochloride (GP 11). To a stirred solution of 3-aminobenzonitrile (4.72 g, 0.04 mol) in benzene (100 mL) was added phenyl isocyanate (5.00 g, 0.042 mol) and the reaction refluxed for 5 h and further stirred at room temperature overnight. The separated white solid was filtered out and recrystallised from methanol (8.2 g, 84 %).

Dry HCl was passed through a cooled solution of the cyano urea (4.00 g, 0.016 mol) and dry methanol (2.7 g, 0.08 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction stored in a refrigerator for 14 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained, as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (4 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white solid of the title compound (2.1 g, 55 %); mp 252-254 °C. ^{1}H NMR (DMSO-d_6) δ: 6.98 (t, 1H); 7.20-7.35 (m, 3H); 7.40-7.55 (m, 3H); 7.70 (d, 1H); 7.95 (s, 1H); 9.1 (bs, 2H); 9.32 (bs, 2H); 9.45 (bs, 1H); 9.18 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}Cl_1N_4O$: C, 57.83; H, 5.20 Cl, 12.19; N, 19.27. Found: C, 57.74; H, 5.20; Cl, 12.16; N, 19.21.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (GP 12). This compound was prepared using the procedure described for compound **GP 9** and replacing benzyl alcohol with phenyl ethyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 2.88 (t, 2H); 3.69 (t, 2H); 4.60 (s, 2H); 7.15-7.30 (m, 5H); 7.49 (d, 2H); 7.80 (d, 2H); 9.17 (bs, 2H); 9.37 (bs, 2H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O$: C, 66.09; H, 6.59; Cl, 12.19; N, 9.63. Found: C, 66.18; H, 6.61; Cl, 12.09; N, 9.56.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (GP 13). This compound was prepared using the

procedure described for compound **GP 11** and substituting 4-phenoxyphenyl isocyanate for phenyl isocyanate. ^{1}H NMR (DMSO-d_6) δ: 6.91-7.20 (m, 4H); 7.10 (t, 1H); 7.30-7.40 (m, 3H); 7.45-7.58 (m, 3H); 7.70(d, 1H); 7.95 (s, 1H); 9.00 (s, 2H); 9.34 (s, 1H); 9.36 (s, 2H); 9.55 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.75$ H_2O: C, 60.60; H, 5.17; Cl, 8.96; N, 14.14. Found: C, 60.62; H, 5.19; Cl, 8.96; N, 14.05.

(4-Amidinobenzyl)-3-phenylpropyl ether hydrochloride (GP 14). A solution of 3-phenylpropyl alcohol (2.72 g, 0.02 mol) in dry THF (20 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (3.92 g, 0.02 mol) in dry THF (20 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. The solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.295 g, 31 %); mp 76-78 °C. ^{1}H NMR (DMSO-d_6) δ: 1.84 (m, 2H); 2.62 (t, 2H); 3.45 (t, 2H); 4.55 (s, 2H); 7.10-7.90 (aromatic, 9H); 9.20 (bs, 2H); 9.45 (bs, 2H). Anal. Calcd. for $C_{17}H_{21}Cl_1N_2O$: C, 66.99; H, 6.94 Cl, 11.63; N, 9.19. Found: C, 67.05; H, 6.95; Cl, 11.54; N, 9.20.

(4-Amidinobenzyl)-3-phenoxylbenzyl ether hydrochloride

(GP 15). This compound was prepared using the procedure described for compound **GP 14** and replacing 3-phenylpropyl alcohol with 3-phenoxylbenzyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 4.55 (s, 2H); 4.63 (s, 2H); 6.95-7.80 (aromatic, 13H); 9.16 (bs, 2H); 9.40 (bs, 2H). Anal. Calcd. for $C_{21}H_{21}Cl_1N_2O_2$: C, 68.38; H, 5.74; Cl, 9.61; N, 7.59. Found: C, 68.20; H, 5.78; Cl, 9.48; N, 7.44.

G-33-650

Annual Report

PROTEASE INHIBITORS AS ANTIVESICANTS

JAMES C. POWERS

School of Chemistry
Georgia Institute of Technology
Atlanta, GA 30332

March 15, 1990

Supported by

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REPORT DOCUMENTATION PAGE

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Powers, James C.

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16. SUPPLEMENTARY NOTATION

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FIELD	GROUP	SUB-GROUP	Antivesicants, Protease Inhibitors, Serine Protease, Sulfur Mustard

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

(SEE BACK OF PAGE)

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19. ABSTRACT

Sulfur mustard is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis. The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister. A total of 26 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

Annual Report

PROTEASE INHIBITORS AS ANTIVESICANTS

JAMES C. POWERS

School of Chemistry
Georgia Institute of Technology
Atlanta, GA 30332

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FOREWORD

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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James C. Powers 3/6/1992

James C. Powers DATE

ABSTRACT

Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic (protein degrading) enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis (outer non-vascular layer of skin). The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister.

The goal of this research program is the design and synthesis of inhibitors for the skin proteolytic enzymes involved in blistering for use as antivesicants. A total of 26 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The structural classes include 7 isocoumarins, 6 phosphonates, 1 benzoxazinone, 5 saccharins, 3 misc. compounds, and 4 derivatives of *p*-guanidino benzoic acid. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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BACKGROUND

Sulfur Mustard. Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. Antimustard ointments which attempt to inactivate the free mustard are ineffective since the mustard quickly reacts with components in the skin and other tissues. Therefore, an effective therapy for sulfur mustard exposure must be based on reversing the physiological processes that result upon contact with this potent vesicant (Cullumbine, 1947).

Bis-(2-chloroethyl)sulfide is a potent alkylating agent which can react with the heterocyclic bases in nucleic acids (Ludlum et al., 1984) and with a wide variety of the side chain functional groups (thiols, thioethers, carboxyl groups, amino groups, imidazole rings, etc.) found in proteins. The majority of the sulfur mustard is secreted in the form of various metabolites such as thiodiglycol, but some is carried by the circulation to other organs, and a significant portion is stored in skin reservoirs (Klain and Bonner, 1987). While the most significant mustard induced injury occurs in the skin (vesication and inflammation), significant numbers of mustard casualties have ocular injuries and cornea impairment for 2-4 months. In cases of severe exposure, there is serious lung and bone marrow damage which results in death.

Mechanism of Sulfur Mustard Induced Blistering. The molecular mechanisms by which sulfur mustard causes toxicity are unknown but mustard is a powerful alkylating agent of DNA and RNA. Papirmeister has suggested that the alkylated purine bases in DNA are unstable and undergo both spontaneous and enzymatic depurination (Papirmeister et al., 1985). This results in DNA strand breaks, and activation of nucleases and other DNA repair mechanisms. As a result, poly(ADP-ribose)polymerase is activated, NAD^+ is depleted, glycolysis is inhibited, and the hexose monophosphate shunt is stimulated (Meier et al., 1987). This causes the release of potent proteolytic enzymes which produces the observed pathology of basal cell necrosis and vesication.

Evidence for the Papirmeister hypothesis includes the isolation and structural characterization of several DNA alkylation products upon treatment of DNA with sulfur mustard (Benschop et al., 1989) and the demonstration of single strand breaks in the DNA after exposure of keratinocyte cultures to low levels of sulfur mustard (Bernstein et al., 1989). In addition, other agents which result in DNA damage such as UV light and radiation have been shown to stimulate the synthesis or release of proteases in fibroblast cultures (Miskin and Reich, 1980).

Proteases are normally controlled by natural plasma protein protease inhibitors such as α_1-protease inhibitor, α_1-antichymotrypsin, and α_2-macroglobulin. If this antiprotease screen is destroyed tissue destruction results. Several of the plasma serpins (serine protease inhibitors) including α_1-protease inhibitor and α_1-antichymotrypsin have essential methionine residues and are susceptible to inactivation by oxidizing agents or alkylating agents. A single dose of sulfur mustard in the mouse brain has recently been shown to cause a burst of oxidants (Elsayed et al., 1989). The serpin screen could then be removed directly by sulfur mustard alkylation or indirectly by oxidation as a result of this oxidative burst. Thus, sulfur mustard exposure probably results both in the release of powerful proteolytic enzymes and in the partial destruction of the protease inhibitor screen which would normally protect the organism from proteolysis.

Other Blistering Disease States Involve Proteases. Blistering disease states which have been described include dermatitis herpetiformis (DH), bullous pemphigoid (BP), chronic bullous disease of childhood, and pemphigus vulgaris. These diseases are characterized by destruction of various connective tissue components of the epidermis or dermis followed by tissue separation and the formation of fluid-filled blisters. Blister fluids from patients with all of these diseases have been shown to contain proteases including elastase and collagenase (Oikarinen et al., 1983). Human polymorphonuclear leukocyte elastase is the major enzyme in DH fluid, while BP fluid predominantly contains the metalloprotease collagenase. A trypsin-like enzyme and a thiol protease have also been implicated in blister formation respectively in recessive dystrophic epidermolysis bullosa and epidermolysis bullosa simplex (Takamori et al., 1985). Incubation of normal human skin with the blister fluid from patients with epidermolysis bullosa letalis, a severe and usually fatal congenital blister disease, results in dermal-epidermal separation. A number of common serine protease inhibitors prevented the separation (Matsumoto and Hashimoto, 1986).

Proteases are Associated with Inflammation. Proteases are important mediators and modulators of inflammation and have been demonstrated in non-blistering inflammatory disease states such as psoriasis and arthritis. The most abundant enzymes are the serine proteases elastase and cathepsin G (a chymotrypsin-like enzyme) from leukocytes; chymases (chymotrypsin-like enzymes), and tryptases (trypsin-like) enzymes from mast cells; plasminogen activator; and the metalloprotease collagenase from leukocytes. These enzymes are capable of cleaving a variety of connective tissue proteins including elastin, collagen, proteoglycans, and other basement membrane components.

Sulfur Mustard Induced Inflammatory Lesions Contain Proteases and Protease-Inhibitor Complexes. The proteolytic enzymes released upon exposure to sulfur mustard have not yet been isolated or characterized, but likely candidates include chymases

and tryptases from mast cells, elastase and cathepsin G from leukocytes, plasminogen activator, and collagenase. Culture fluids from mustard-induced inflammatory lesions in rabbit skin show 3 to 6 fold increased levels of proteases both in developing and healing lesions (Higuchi et al., 1987). These fluids will hydrolyze two synthetic peptide substrates, Boc-Leu-Gly-Arg-AFC (Boc = *t*-butyloxycarbonyl, AFC = 7-amino-4-trifluoromethyl coumarin) and Bz-Phe-β-naphthyl ester (Bz = benzoyl). The first peptide is a substrate for trypsin, tryptases, plasmin, plasminogen activator and other trypsin-like enzymes, while the latter is a substrate for chymotrypsin-like enzymes including chymases and cathepsin G. The rabbit skin culture fluids did not consistently hydrolyze four other synthetic peptide substrates (two for elastase and two for cathepsin G) or the protein elastin (elastase's natural substrate). Exposure of human skin in culture to sulfur mustard results in a 41 % increase in plasminogen activator activity (Dannenberg et al., 1989), an enzyme which is known to be associated with blister formation (Hashimoto et al., 1983). The enzymatic activity of chymases, tryptases, and angiotensin converting enzyme toward small synthetic substrates were not elevated.

The proteases found in the culture fluids from mustard-induced inflammatory lesions in rabbit skin are not present as free active enzymes, but are found as inactive complexes with their natural plasma protease inhibitors α_1-protease inhibitor and α_2-macroglobulin (Harada et al., 1987; Dannenberg et al., 1987; Higuchi et al., 1987). These complexes are formed as natural protein protease inhibitors from the plasma react with the proteases being released at the site of inflammation. The protease-inhibitor complexes are incapable of hydrolyzing protein substrates and complex formation thus protects the organism from further damage. The natural plasma protease inhibitors are probably not completely destroyed by exposure to low levels of sulfur mustard and are still available to react with some of the proteases released in the blister.

Protease-inhibitor complex formation hinders the identification of the proteases present in sulfur mustard induced culture fluid. Complexes of elastase and cathepsin G respectively with α_1-protease inhibitor and α_1-antichymotrypsin are inactive toward both synthetic peptide substrates and natural protein substrates. Thus the lack of hydrolysis of elastin or elastase substrates does not preclude the presence of inactivated elastase in the culture fluids. Similarly the lack of hydrolysis of the protein fibrin by the culture fluids doesn't exclude the presence of plasmin or plasminogen activator. In summary, it is now clear that there is a chymotrypsin-like enzyme (chymase), a trypsin-like enzyme (tryptase), and plasminogen activator in the sulfur mustard induced inflammatory lesions, but the presence of other enzymes has not been excluded.

Skin Serine Proteases Have Been Isolated and Characterized. The dermis of human skin is a rich source of

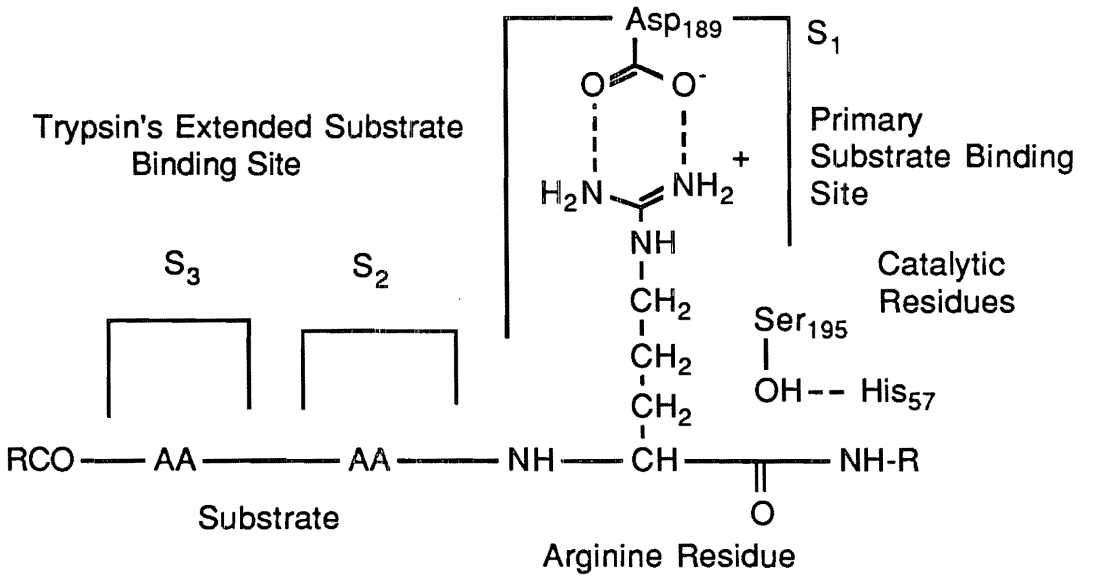
mast cells and salt extraction of human skin has yielded two serine proteases, a chymase and a tryptase. These serine proteases are localized in the granule fraction of mast cells, a cell type which is located predominantly in connective tissue. The chymase has been demonstrated immunocytochemically to bind to the dermo-epidermal junction in skin (Sayama et al., 1987). Both the mast cell chymase and tryptase are able to specifically cleave proteins found in the dermal-epidermal boundary and cause vesication. The chymase is incompletely inhibited by plasma due to a 650 fold slower rate of reaction with the serpins α_1-protease inhibitor and α_1-antichymotrypsin (Schechter et al., 1989), while the tryptase appears not to be inhibited by most protein protease inhibitors (Schechter et al., 1983). This may explain the ready detection of chymase and tryptase activity in culture fluids from mustard-induced lesions in rabbit skin.

Rat mast cells contain two chymases (RMCP I and RMCP II) which have been more extensively characterized that the chymase from human skin. Both of these serine proteases are highly homologous to human mast chymase and human leukocyte cathepsin G. RMCP I and RMCP II have both been sequenced and the x-ray crystal structure of RMCP II has been determined, while the x-ray structure of RMCP I is underway (Woodbury and Neurath, 1980; Remington et al., 1988). The sequence of dog mast cell tryptase and a related dog mast cell protease have been determined by gene sequencing techniques (Vanderslice et al., 1989) and it is likely that the sequences for human skin tryptase and chymase will be available in the next few years.

The substrate specificity and inhibition profile of human skin chymase, human skin tryptase, RMCP I, RMCP II, and related enzymes have been studied in the laboratory of the principle investigator (Powers et al., 1985). Human skin chymase hydrolyzes peptide substrates containing aromatic amino acid residues and prefers Phe-AA and Tyr-AA bonds over Trp-AA bonds (AA = any amino acid residue) in contrast to chymotrypsin which prefers Trp over Phe and Tyr. One of the best peptide substrates is Suc-Phe-Val-Pro-Phe-NA (Suc = succinyl, NA = 4-nitroanilide). Human skin tryptase is a trypsin-like enzyme, but seems to prefer double basic residues in its substrates (Tanaka et al., 1983). For example, the thioester substrate Z-Lys-Arg-SBu-i (Z = benzyloxycarbonyl, SBu-i = thioisobutyl ester) is hydrolyzed by human skin tryptase with a k_{cat}/K_M = 59,000,000 $M^{-1}s^{-1}$, a second order rate constant which is close to the diffusion controlled rate.

Serine Protease Specificity. The specificity of serine proteases toward natural peptide substrates or synthetic inhibitors is determined by the nature of the primary substrate specificity pocket (S_1) and secondary subsites (S_2, S_3, etc.) on the surface of each individual enzyme. Trypsin's primary specificity site contains an Asp residue in the back of the S_1 pocket so that trypsin will only bind to and hydrolyze peptide

substrates containing lysine or arginine residues (a schematic model of trypsin with a bound substrate is shown below). The three-dimensional structure of chymotrypsin is quite similar except that the Asp-189 in trypsin is replaced by Gly-189 in chymotrypsin. As a result the S_1 pocket of chymotrypsin in very hydrophobic and chymotrypsin prefers substrates containing aromatic amino acid residues such as Trp, Tyr, and Phe. With many serine proteases, interactions of inhibitors with the extended substrate binding site (S_2, S_3, etc.) are important to increase the specificity and reactivity of the inhibitor. This is clearly the case with human skin chymase and tryptase. For example, interaction of the Lys in the substrate Z-Lys-Arg-SBu-i with the S_2 subsite of human tryptase results in an accelerated rate of hydrolysis, while little change in hydrolysis rate is observed with trypsin.



HYPOTHESIS

It is clear—no matter the exact mechanism of their release or their source—that proteases are major factors in the tissue destruction that accompanies mustard induced vesication. We propose that protease inhibitors will be effective antivesicants and should be useful both in preventing blistering and in the treatment of blisters. Appropriate target proteases are the mast cell chymase and tryptase, serine proteases which are localized in the skin and have the ability to cleave proteins at the dermal-epidermal junction. However other serine protease such as elastase and cathepsin G from leukocytes, and plasminogen activator may also be involved. Evidence for the involvement of other classes of proteases such as the metalloprotease collagenase or the thiolprotease cathepsin B is incomplete or lacking at present, although the mast cell tryptase is able to activate latent collagenase (Gruber et al., 1989).

Research Strategy. Since the exact target enzyme (or enzymes) is not known with certainty, we have decided to synthesize general serine protease inhibitors, specific chymase inhibitors, specific tryptase inhibitors, specific plasminogen activator inhibitors, and specific inhibitors for other enzymes as appropriate. We plan to shift our emphasis to a particular enzyme or group of enzymes when more biological data is obtained on the role of specific proteases in blistering or when we receive animal test data on the compounds which we have already submitted. Until that time, we plan to submit for testing a wide variety of potentially active structures including both general serine protease inhibitors and inhibitors which are more enzyme specific.

PROGRESS REPORT

Research Goals

1. Prepare and submit for animal testing 3-5 inhibitors of serine proteases such as 3,4-dichloroisocoumarin and saccharins each year.

2. Prepare and submit for testing 3-5 inhibitors of human skin chymase each year.

3. Prepare and submit for testing 3-5 inhibitors of human skin tryptase each year.

4. Prepare and submit for testing each year 3-5 inhibitors for other human serine proteases-such as human leukocyte elastase, cathepsin G, and plasminogen activator-which may have a role in vesication.

5. Assay all inhibitors with human skin serine proteases and related enzymes for *in vitro* effectiveness.

Research Progress-Summary

1. A total of 26 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing during the first year of the contract. The structural classes include 7 isocoumarins, 6 phosphonates, 1 benzoxazinone, 5 saccharins, 3 misc. compounds, and 4 derivatives of guanidino benzoic acid. All the inhibitors submitted are listed below in the section on biological testing of the inhibitors.

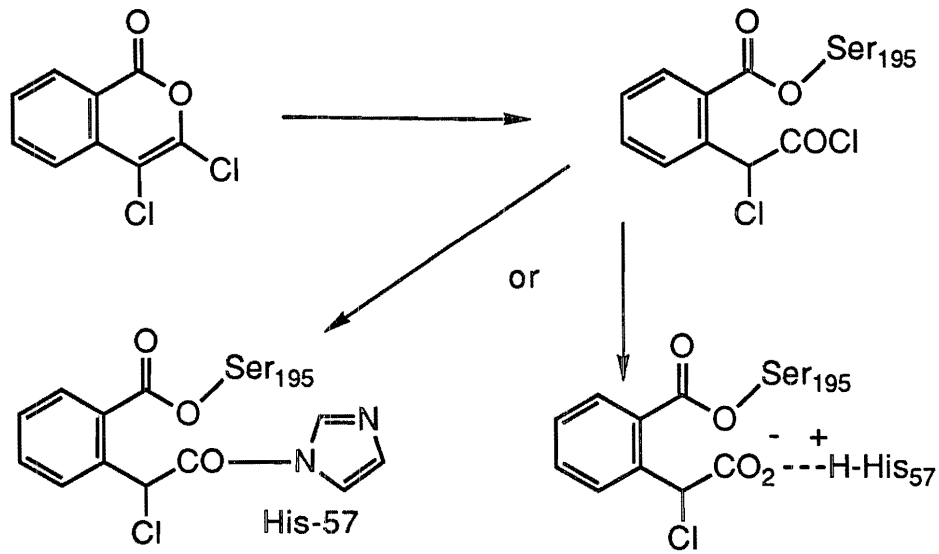
2. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

Inhibitors Submitted.

Isocoumarins-General Inhibitors. Dichloroisocoumarin (BL58572) is an excellent general inhibitor of serine proteases and was discovered in the laboratory of the principal investigator (Harper et al., 1985). With the exception of the bacterial enzyme subtilisin, 3,4-dichloroisocoumarin is an inactivator of all serine proteases which have been tested, including human leukocyte elastase, human skin chymase, dog skin chymase, rat mast cell protease I, and rat mast cell protease II.

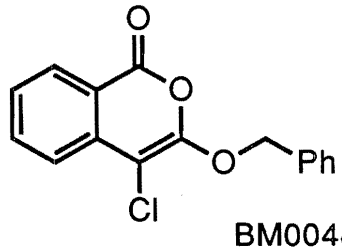
The dichloroisocoumarin ring system contains a masked acid chloride (or ketene) functional group which is exposed when an acyl enzyme is formed upon reaction with the active site serine of

a serine protease (Harper et al., 1985). The acyl enzyme (top right of figure) which is formed initially can react further by acylating the active site histidine to form a doubly acylated enzyme derivative (bottom left) or can hydrolyze to form an acyl enzyme stabilized by a salt link between the protonated histidine and the inhibitor carboxyl group (bottom right). The monochloro derivative, 3-chloroisocoumarin (BL57637), inhibits chymotrypsin-like enzymes at slower rates than 3,4-dichloroisocoumarin and does not touch trypsin. The acyl enzymes formed upon reaction with dichloroisocoumarin have variable stabilities, but in general the half-lives for reactivation (deacylation) are greater than 8 hrs at pH 7.5.

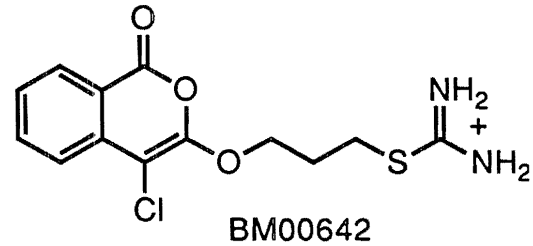


Dichloroisocoumarin and 3-chloroisocoumarin are formed by reaction of homophthalic acid with PCl_5.

Isocoumarins-Specific Inhibitors. We have also synthesized a number of isocoumarin inhibitors which are more specific for the active sites of chymases or tryptases. Inhibitors targeted for chymase should contain an aromatic side chain which resembles the side chain of Phe, Tyr or Trp, while those inhibitors targeted for tryptase should containing a charged group which resembles the side chain of Arg or Lys. Several of the more specific isocoumarin inhibitors are shown below. Inhibitors with the benzyloxy (such as BM00482) or phenylethoxy groups (BL57413) were targeted at the chymases, while those with basic side chains (such as BM00642) were targeted at the tryptases.

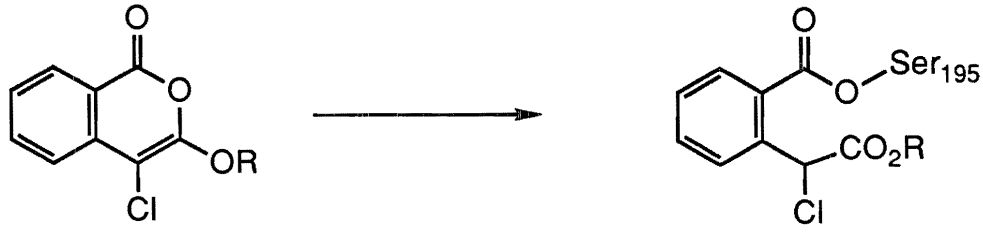


BM00482

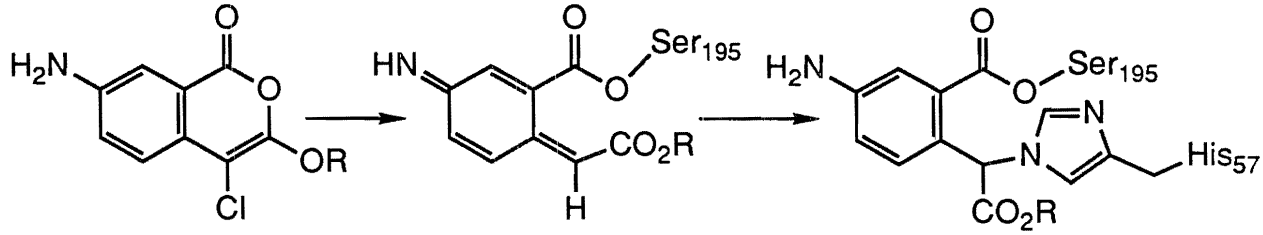


BM00642

The mechanism of inhibition of serine proteases by 3-alkoxy-4-chloroisocoumarins involves acylation of the active site serine-195 to form acyl enzymes with varying stabilities ($t_{1/2}$ = hrs to days) depending on the nature of the alkoxy group (Harper and Powers, 1985).

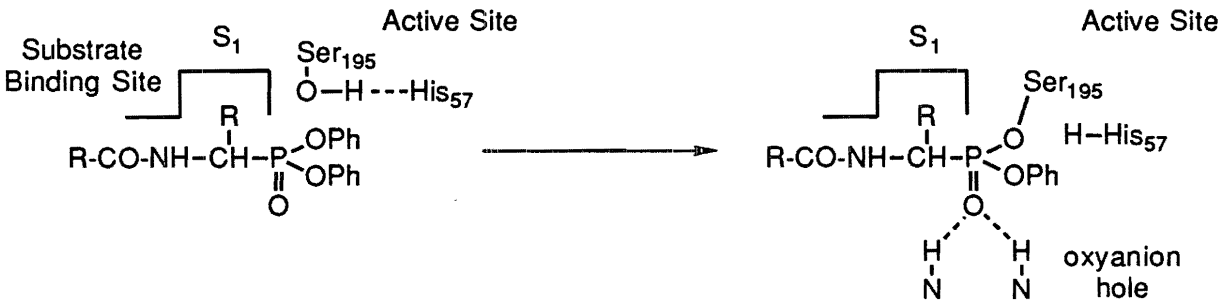


Analogous isocoumarins with electron donating substituents in the 7-position such as 3-alkoxy-7-amino-4-chloroisocoumarins (below) are mechanism-based or suicide inhibitors of serine proteases (Powers et al., 1989). These inhibitors also acylate serine proteases, but form stable acyl enzymes which are not reactivated upon long standing or upon treatment with hydroxylamine. The inhibition mechanism involves formation of an acyl enzyme which can then eliminate chloride to form a quinone imine methide (center). This intermediate then irreversibly alkylates His-57 with the formation of a stable covalent bond between enzyme and inhibitor. This mechanism is supported by x-ray crystallographic studies of complexes of isocoumarin inhibitors bound to the active site of porcine pancreatic elastase (Bode et al., 1989). Thus far, four separate isocoumarins have been studied crystallographically, two give simple acyl enzyme structures (above) and two give acyl enzyme structures where His-57 has been alkylated.



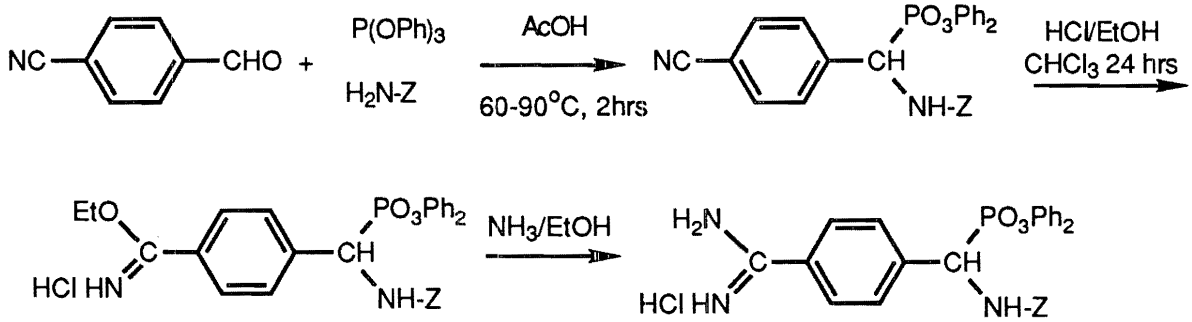
Isocoumarins which have been submitted and should inhibit serine proteases by the above mechanism include 7-amino-4-chloro-3-(cyclohexylmethoxy)isocoumarin and 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin (BM01096).

α-Aminoalkylphosphonates Diphenyl Esters. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations (Oleksyszyn and Powers, 1989). These peptide derivatives phosphonylate the active site serine to form stable phosphonyl derivatives. Good interactions with the S_1 pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative.



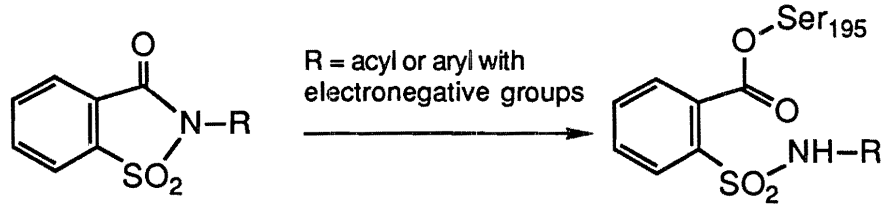
Phosphonate diphenyl ester inhibitors are chemically stable, relatively easy to synthesize, do not react with acetylcholinesterase, form very stable derivatives possibly due to their resemblance to the tetrahedral intermediate involved in peptide bond hydrolysis, and have considerable potential utility as therapeutic agents.

We have submitted a number of simple amino acid and peptide derivatives of phosphonate diphenyl esters including Z-Met^P(OPh)$_2$ (BL57646), Z-Val^P(OPh)$_2$ (BL57968), Z-Phe^P(OPh)$_2$ (BL57422), Z-Phe-Phe^P(OPh)$_2$ (BL57842), and Suc-Val-Pro-Phe^P(OPh)$_2$ (BL59382). More recently, we have accomplished the synthesis of an amidinophenyl phosphonate derivative by the route shown below. The benzyloxycarbonyl (Z) derivative has been submitted recently for testing. We have also synthesized a few peptide derivatives of the amidinophenyl phosphonate, but not in sufficient quantities for submission.

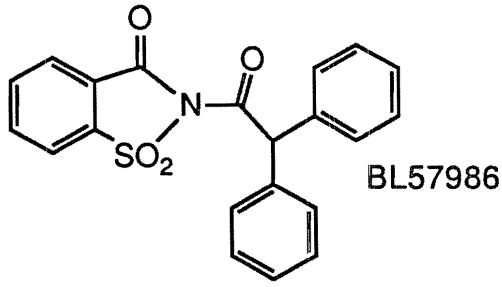


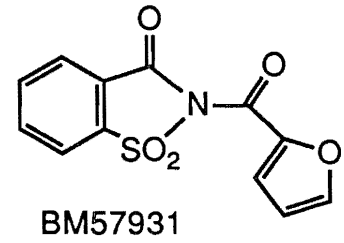
Saccharin Inhibitors. N-Acyl and N-aryl saccharins are potent acylating agents of HL elastase, cathepsin G, and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981). A few

of the N-acyl derivatives such as N-furoyl, N-thienoyl, and N-benzoylsaccharin inhibit trypsin with IC_{50} values of 0.7-2.4 μM. These structures were initially designed as acyl transfer reagents, but studies using ^{35}S-labeled N-furoylsaccharin indicated that the saccharin portion of the inhibitor becomes covalently and stoichiometrically bound to both HL elastase and pancreatic elastase upon acylation.

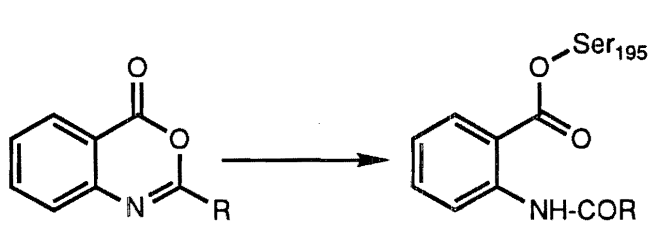


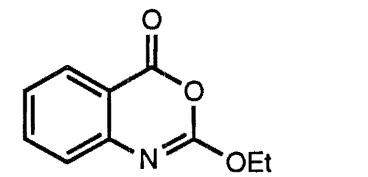
Two of the saccharins which we have submitted are shown below.


BL57986


BM57931

Benzoxazin-4-ones. Substituted benzoxazin-4-ones were discovered to be potent inhibitors of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, cathepsin G, and chymotrypsin by the PI (Teshima et al., 1982). Mechanistic studies by Abeles showed that these compounds were forming stable acyl enzyme derivatives (shown below) with chymotrypsin (Hedstrom et al., 1984) and this has been confirmed by x-ray crystallographic studies with two benzoxazinones bound to PP elastase (Radhakrishnan et al., 1987). Due to the potential of benzoxazinone inhibitors of HL elastase for treatment of emphysema, a group at Syntex Canada has synthesized over 100 new benzoxazinones, carried out a structure-function study as substituents were varied on the ring system, and studied the plasma stability of these compounds (Spencer et al., 1986; Krantz et al., 1987; Krantz et al., 1990). Thus far, we have submitted only one benzoxazinone, BM00491, a good elastase inhibitor.

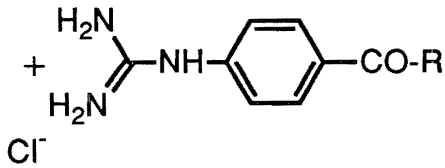




2-Ethoxy-4H-3,1-benzoxazin-4-one

BM00491

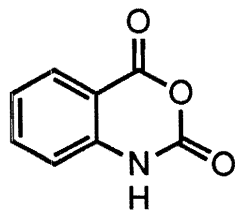
Guanidinobenzoic Acid Inhibitors. Esters of *p*-guanidinobenzoic acid have been reported to be potent inhibitors for various trypsin-like enzymes (Okutome et al., 1984; Fujii et al., 1977) and *p'*-nitrophenyl-*p*-guanidinobenzoate is widely used as an active-site titrant for these enzymes (Chase and Shaw, 1970). An active-site titrant for trypsin-like enzymes developed in the laboratories of the PI is benzyl *p*-guanidinothiobenzoate (Cook and Powers, 1983). We have submitted samples of four *p*-guanidinobenzoic acid derivatives for testing.



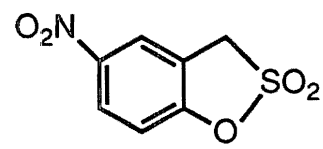
R = OH (BM01363), OEt (BM01185),

NH-Val-NH_2 (BM02655), OCH_2CONH_2 (BM03143)

Miscellaneous Inhibitors. Two heterocyclic general serine protease inhibitors have been submitted. They are isatoic anhydride which has been shown to acylate the active site of chymotrypsin and form a stable acyl enzyme (Moorman and Abeles, 1982) and 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide which forms a stable sulfonyl derivative also with chymotrypsin.

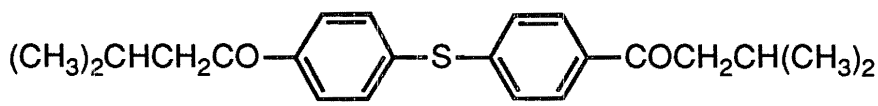


Isatoic Anhydride
BL57646



5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide
BM00491

The sulfide shown below is an effective inhibitor of elastase which was discovered in the laboratory of the PI.

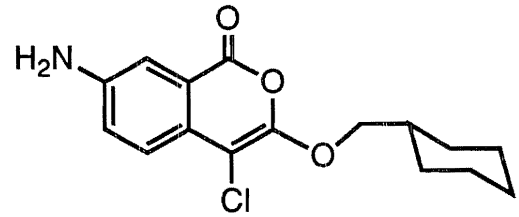


BL57940

Biological Test Data.

All the inhibitors which we have synthesized have been tested for inhibitory potency against a variety of serine proteases. Kinetic data obtained with the various inhibitors are shown in Tables I-IV. Most of the inhibitors are irreversible or slowly reversible inhibitors and we report the second order inhibition rate constants $k_{obs}/[I]$. Several of the inhibitors reported in the tables have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. A second order inhibition rate constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing the enzyme and inhibitor in the assays). The half-life of the inhibition reaction can be calculated from the equation $t_{1/2} = 0.693/\{[I]*k_{obs}/[I]\}$. Thus, an inhibitor with a $k_{obs}/[I]$ value of 100,000 $M^{-1}s^{-1}$ would have an inhibition half-life of 6.93 sec. at an inhibitor concentration of 1 μM, while an inhibitor with $k_{obs}/[I]$ = 10,000 would have a half-life of 69 sec. For the few reversible inhibitors investigated, K_I values (dissociation constant of the enzyme-inhibitor complex) or IC_{50} values are given.

The data with bovine chymotrypsin, cathepsin G, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The best isocoumarin inhibitor in this table is 3-benzyloxy-4-chloroisocoumarin (BM00482) with a $k_{obsd}/[I]$ = 12,000 $M^{-1}s^{-1}$ for the human skin chymase. Increasing the length of the side chain at position 3 by one methylene group [4-chloro-3-(2-phenylethoxy)isocoumarin, BL57413] reduces the activity by a factor of 35. Thus far, we have been unable to synthesize the 7-amino derivative of BM00482 which would allow the synthesis of many additional acylamino analogs which we would expect to be better inhibitors due to increased interaction with the extended substrate binding site of chymases. We synthesized the 7-nitro derivative, but during attempted reduction of the nitro group, cleavage of the benzyloxy functional group took place concurrently with reduction of the nitro group. In order to avoid this problem we synthesized the 3-cyclohexylmethyloxy derivative shown below which contains a cyclohexane ring in place of the phenyl in the 3-benzyloxy derivative. Unfortunately this modification resulted in a much weaker inhibitor and the compound has $k_{obsd}/[I]$ = 25 $M^{-1}s^{-1}$ with human skin chymase.



The best phosphonate inhibitor for chymotrypsin-like enzymes is Suc-Val-Pro-Phe^{P}(OPh)$_2$, which corresponds to the sequence of an excellent 4-nitroanilide substrate for these enzymes. NMR studies with chymotrypsin indicate that only one of the two stereoisomers reacts with the enzyme ($k_{obsd}/[I]$ = 146,000 $M^{-1}s^{-1}$ calculated for

the single isomer, the value in the table is for the DL mixture). The ^{31}P NMR of chymotrypsin inhibited by this peptide phosphonate shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonylated enzyme derivative. The tripeptide phosphonate Suc-Val-Pro-Phe^{P}(OPh)$_2$ makes better interactions with the extended substrate binding site of the enzyme than is possible with the shorter dipeptide or amino acid phosphonate derivatives.

All of the saccharins submitted so far have high $k_{obsd}/[I]$ values with the various chymotrypsin-like enzymes and low IC_{50} values with the elastases tested. One of the better inhibitors in this family is N-furoylsaccharin. The acyl enzymes formed upon acylation of serine proteases by acyl saccharins have variable stabilities. Furoyl saccharin and benzoyl saccharin form inhibited elastase derivatives which are very stable and have half-lives for deacylation of 80-160 hrs. In contrast the chymotrypsin derivatives have much shorter half-lives in the range of 1.9 hrs. One disadvantage of some acyl saccharins is their fairly rapid hydrolysis at neutral pH values.

The data with porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE) is given in Table II. All the isocoumarin derivatives reported in this table are excellent inhibitors of HLE, the best one being the 7-ureido derivative BM01096 with a $k_{obsd}/[I] = 140,000\ M^{-1}s^{-1}$ for HLE. It is also highly selective compared with 3,4-dichloroisocoumarin and 3-chloroisocoumarin which is a logical consequence of the improvement in binding resulting from substitution at the 7-amino group. We have also submitted an excellent benzoxazinone inhibitor for HLE (BM00651) and analogs of this derivative will be made in the future.

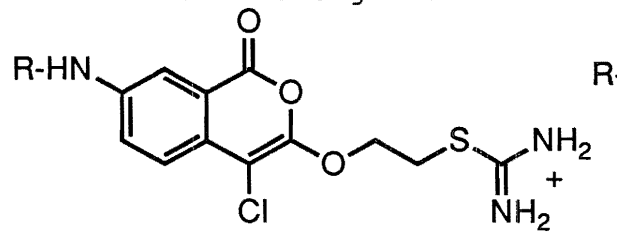
The phosphonate derivatives which we have submitted thus far are not good inhibitors for PPE or HLE. This is not surprising since the sequence of the tripeptide was chosen for chymase inhibition and this sequence is very specific for the chymases as discussed earlier. An excellent phosphonate inhibitor for the elastases has been synthesized only in a small scale. This inhibitor, Boc-Val-Pro-Val^{P}(OPh)$_2$, has $k_{obsd}/[I]$ value of 27,000 $M^{-1}s^{-1}$ for human leukocyte elastase (HLE) and 11,000 $M^{-1}s^{-1}$ for porcine pancreatic elastase (PPE). Again this sequence corresponds to a good HLE substrate sequence and at low concentrations this peptide did not react with chymotrypsin.

The inhibition data obtained with bovine trypsin, human lung tryptase, rat skin tryptase, human skin tryptase, and human recombinant tissue plasminogen activator is given in Table III. The best tryptase inhibitor submitted is the 3-(isothioureidopropoxy)isocoumarin BM00642 with a $k_{obs}/[I] = 650,000$ $M^{-1}s^{-1}$ for the rat skin tryptase, an extremely rapid inhibition rate. However, the acyl-enzyme formed with this inhibitor is unstable and the enzyme regains its activity within 5 min. We plan to synthesize analogs of this inhibitor which should form

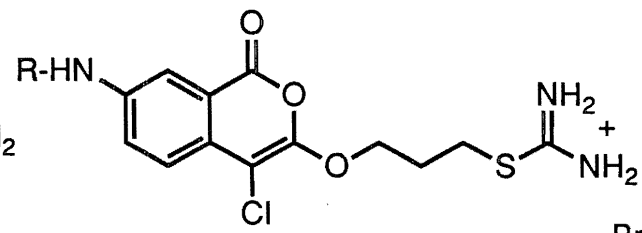
more stable acyl enzyme derivatives and thus be longer acting inhibitors.

Several *p*-guanidinobenzoic acid derivatives have been tested as inhibitors for the various tryptases. One of these derivatives, O-(*p*-guanidinobenzoyl)glycolamide is an excellent inhibitor of the rat skin tryptase, but is a much poorer inhibitor of human skin tryptase. In contrast to the isocoumarin BM00642, the inhibited derivative did not regain enzyme activity upon standing. Thus, we plan to pursue this lead and synthesize additional derivatives in the near future. Our goals will be improvement of the inhibitory potency toward the human skin tryptase while maintaining the stability of the acyl enzyme derivatives which are formed.

Table IV lists the inhibition data with bovine trypsin, human lung tryptase, rat skin tryptase, human skin tryptase, and human recombinant tissue plasminogen activator by the isocoumarin isothiureido derivatives depicted below. The CiTEtOIC derivatives are based on 4-**c**hloro-3-(**iso**thiourei**do**e**th**oxy)**iso**coumarin and the CiTPrOIC derivatives are based on 4-**c**hloro-3-(**iso**thiourei**do**p**r**opoxy)**iso**coumarin. These inhibitors were synthesized in the course of another research project and were tested with the tryptases in order to obtain information about the nature of the active site of the tryptases which will be useful for our future design work.



R-NH-CiTEtOIC



R-NH-CiTPrOIC

R = H, alkyl-CO, arylalkyl-CO, AA-CO (amino acyl), aryl-NHCO, arylalkyl-NHCO

The following correlations can be made from the data reported in Table IV: a) the propoxy derivatives (RNH-CiTPrOIC) are better inhibitors for the rat skin tryptase while the ethoxy derivatives (RNH-CiTEtOIC) are better inhibitors for the human tryptases and recombinant tissue plasminogen activator; b) acyl derivatives were weaker inhibitors of the rat skin tryptase and human recombinant tissue plasminogen activator and equally potent for the human lung tryptase; c) an extra CH_2 in the urea side chain results in better inhibitors for rat skin and human lung tryptases while shorter chains are preferred by human recombinant tissue plasminogen activator; d) longer chains on the simple acyl derivatives increase inhibitory potency toward all the tryptases and e) N-acyl substitution of a D-Phe results in greater inhibitory potency for all the tryptases when compared to substitution by L-Phe.

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
		ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Isocoumarin Inhibitors				
BL58572	3,4-dichloroIC[g]	570	28	580	27	82
BL57637	3-chloroIC[g]	330	24	85		
BL57413	4-chloro-3-(2-phenylethoxy)IC	3800	100	200	340	
BM00482	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	39,000
	7-amino-4-chloro-3-cyclohexylmethoxyIC				25	
		Miscellaneous Inhibitors				
BL57646	isatoic anhydride	580	114	250		
BL57940	di(4-isovaleroylphenyl)sulfide		NI[h]	25%[i]		
		Phosphonate Inhibitors				
BL57959	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
BL57968	Z-Val^{P}(OPh)$_2$	0.4	NI[h]	NI[h]		
BL57422	Z-Phe^{P}(OPh)$_2$	260	76	89		
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI[h]	18		
BL59382	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000	190,000	
	Saccharin Inhibitors					
BL57977	N-benzoylsaccharin	15,000	45,000	16,000		
BL57995	N-phenylacetylsaccharin	11,000	31,000	1,300		
BL57986	N-diphenylacetylsaccharin	10,000	14,000	9,800		
BL57931	N-furoylsaccharin	22,000	39,000	20,000		
BM00464	N-cyanomethylsaccharin	NI[h]				

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM; human skin chymase, 0.07

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

μM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), human skin chymase and RMCP II were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Inhibitor concentrations were in the range 5.2 μM-320 μM. N-Cyanomethyl saccharin was inactive at 1 mM.

[c]Inhibitor concentrations were in the range: 4.8 μM-158 μM.

[d]Inhibitor concentrations were in the range: 3-148 μM.

[e]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 45 μM; 7-amino-4-chloro-3-cyclohexylmethoxyIC, 0.44 mM; Suc-Val-Pro-Phe$^{P}(OPh)_2$, 0.54 μM.

[f]Inhibitor concentrations were as follows: 3,4-dichloroisocoumarin, 540 μM; 3-benzyloxy-4-chloroIC, 1.0 μM.

[g]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

[h]No inhibition.

[i]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
BL58572	3,4-dichloroIC[d]	2,500	9,000
BL57637	3-chloroIC[d]	510	3,900
BL57413	4-chloro-3-(2-phenylethoxy)IC		
BM01096	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
	Miscellaneous Inhibitors		
BL57646	isatoic anhydride		
BL57940	di(4-isovaleroylphenyl)sulfide		2 μM[e]
BM00651	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM00491	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Saccharin Inhibitors		
BL57977	N-benzoylsaccharin	5.2 μM[f]	2.4 μM[f]
BL57995	N-phenylacetylsaccharin		
BL57986	N-diphenylacetylsaccharin		
BL57931	N-furoylsaccharin	0.58 μM[f]	0.36 μM[f]
BM00464	N-cyanomethylsaccharin		
	Phosphonate Inhibitors		
BL57959	Z-Met^{P}(OPh)$_2$	NI[g]	0.8
BL57968	Z-Val^{P}(OPh)$_2$	2.5	90

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
BL57422	Z-Phe^{P}(OPh)$_2$	NI	6
BL57842	Z-Phe-Phe^{P}(OPh)$_2$	NI	NI
BL59282	Suc-Val-Pro-Phe^{P}(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25 °C. Enzyme concentrations were: PPE, 1.6 μM; HLE, 0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were in the range: 3-320 μM.

[c]Inhibitor concentrations were in the range: 1.2-230 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

[e]Inhibition was not time dependent and the IC_{50} was obtained.

[f]IC_{50} values obtained from Zimmerman, M., Morman, H., Mulvey, D., Jones, H, Frankshun, R. and Ashe, B. M. (1980) J. Biol. Chem. **255**, 9848-9851.

[g]No inhibition.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid and Phosphonate Inhibitors.

Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Isocoumarin Derivatives					
BL58572	3,4-dichloroisocoumarin	200	190	610		70
BM00642	4-chloro-3-(3-isothioureido-propoxy)isocoumarin	46,000	260,000	650,000	83,000	13,000
	Guanidinobenzoic Acid Derivatives					
BM01363	*p*-guanidinobenzoic acid	NI[g]				
BM01185	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7		NI[g]
BM02655	N-(*p*-guanidinobenzoyl) valine amide	4.4	4.7	1.3	2,000	
BM03143	O-(*p*-guanidinobenzoyl) glycolamide	100	19%[h]	130,000	5.8	NI[g]

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid and Phosphonate Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Phosphonate Inhibitors					
	Z-NHCH(AmPh)PO_3Ph_2[i]	2,000		16		

[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM; human r-t-PA, 0.017 μM Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase, human skin tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; *p*-guanidinobenzoic acid, 0.44 mM; ethyl *p*-guanidinobenzoate, 0.43 mM; *p*-guanidinobenzoyl valine amide,0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 mM.

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 μM; ethyl *p*-guanidinobenzoate, 0.42 mM; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.42 mM.

Table III (Continued).

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25° C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 μM; ethyl *p*-guanidinobenzoate, 0.45 mM ; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 μM.

[e]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.35 μM; *p*-guanidinobenzoyl valine amide, 3.5 μM; O-(*p*-guanidinobenzoyl)glycolamide, 1.7 mM.

[f]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 4.3 μM; ethyl *p*-guanidinobenzoate, 0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.43 mM.

[g]No inhibition.

[h]Inhibition was not time dependent and the % inhibition was measured at 0.42 mM.

[i] AmPh = 4-amidinophenyl

Table IV. Inhibition Rates of Bovine Trypsin and Tryptases by Isothiureido Isocoumarin Derivatives[a].

Compound	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
	Bovine Trypsin[b]	Rat Skin Tryptase[c]	Human Lung Tryptase[d]	Human r-t-PA[e]	Human Skin Tryptase[f]
CiTPrOIC		650,000	260,000	13,000	83,000
7-NH_2-CiTPrOIC	410,000[g]	39,000		13,000	
7-$PhCH_2NHCONH$-CiTPrOIC	51,000	270,000	190,000	18,000	68%[h]
7-PhNHCONH-CiTPrOIC	63,000	250,000	140,000	19,000	38,000
7-CH_3CONH-CiTPrOIC	107,000	99,000	60%	7,000	
7-$PhCH_2CH_2CONH$-CiTPrOIC	88,000	170,000	180,000	15,000	
7-$PhCH_2CONH$CiTPrOIC	165,000	145,000	140,000	9,000	
7-L-PheNH-CiTPrOIC		96,000	54%[h]	11,000	
7-Boc-L-PheNH-CiTPrOIC		150,000	170,000	6,000	
7-PhNHCONH-CiTEtOIC	21,000	170,000	170,000	16,000	
7-$PhCH_2NHCONH$-CiTEtOIC		200,000	280,000	19,000	
7-$PhCH_2CONH$-CiTEtOIC		120,000	110,000	64%[h]	

Table IV (Continued). Inhibition Rates of Bovine Trypsin and Tryptases by Isothiureido Isocoumarin Derivatives[a].

Compound	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
	Bovine Trypsin[b]	Rat Skin Tryptase[c]	Human Lung Tryptase[d]	Human r-t-PA[e]	Human Skin Tryptase[f]
7-D-PheNH-CiTEtOIC		360,000	60,000	15,000	62,000
7-Boc-D-PheNH-CiTEtOIC		135,000		65%[h]	

[a]Inhibition rates were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer for bovine trypsin; 25 mM Phosphate, 0.5 M NaCl, 1mM EDTA, pH 7.5 buffer for rat skin tryptase and 0.1M Hepes, 0.5M NaCl, pH 7.5 for human lung tryptase, human skin tryptase and r-t-PA. All enzymes were assayed with Z-Arg-SBzl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM). Enzyme concentrations were: trypsin, 0.12μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM; r-t-PA, 0.017 μM. Reaction mixtures contained 8% Me_2SO and assays were performed at 25 °C. [b]Inhibitor concentrations were in the range 1.1-4.6 μM. [c]Inhibitor concentrations were in the range 0.35-0.51 μM. [d]Inhibitor concentrations were in the range 0.42-0.47 μM. [e]Inhibitor concentrations were in the range 4.2-5.0 μM. [f]Inhibitor concentrations were in the range 0.34-0.39 μM. [g]Data was obtained from Kam et al., 1988. [h]Inhibition was not time dependent and the % inhibition was measured at inhibitor concentrations in the range 0.34-5.0 μM.

Animal Testing Priorities

The various compounds which we have submitted have been prioritized for animal testing and the following table lists the priority which we have assigned to each compound and the reasons for that priority.

High-1 BL58572 3,4-dichloroisocoumarin
This isocoumarin is a general serine protease inhibitor and effectively inhibits most of the enzymes tested.

High-2 BL59282 Suc-Val-Pro-Phe^{P}(OPh)$_2$
This peptide phosphonate is a reactive and specific inhibitor for chymotrypsin-like enzymes including chymases.

High-3 BM00642 4-chloro-3-(3-isothioureidopropoxy)isocoumarin
This isocoumarin is a very reactive inhibitor for the rat skin tryptase.

High-4 BM00651 2-ethoxy-4H-3,1-benzoxazin-4-one
This benzoxazinone inhibitor is an effective inhibitor for elastase.

High-5 BL57931 N-furoylsaccharin
This saccharin is a general protease inhibitor and inhibits elastases and chymotrypsin-like enzyme quite effectively and is probably a moderate inhibitor for trypsin-like enzymes.

High-6 O-(*p*-guanidinobenzoyl)glycolamide
This guanidinium substituted derivative is an effective inhibitor for trypsin-like enzymes

High-7 BM01096 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin
This isocoumarin is an effective elastase inhibitor.

High-8 BL57940 di(4-isovaleroylphenyl)sulfide
This aromatic derivative is an effective elastase inhibitor.

High-9 BM00482 4-chloro-3-benzyloxyisocoumarin
This isocoumarin is an effective inhibitor for chymotrypsin-like enzymes.

High-10 BL57977 N-benzoylsaccharin
This saccharin is an effective inhibitor for many serine proteases including chymotrypsin-like enzymes and elastases.

EXPERIMENTAL SECTION

4-Chloro-3-(2-phenylethoxy)isocoumarin (BL57413). This compound was prepared according to procedure Harper J. W., and Powers, J. C. (1985) Biochemistry 24, 7200: m.p. 99-100 °C. Anal. Calcd. for $C_{17}H_{13}O_3Cl \cdot 0.5\ H_2O$: C, 65.92; H, 4.52; Cl, 11.46. Found: C, 66.20; H, 4.50; Cl, 11.58.

3-Chloroisocoumarin (BL57637). This compound was prepared according to the procedure of Davis, W., and Poole, H. G.(1928) J. Chem. Soc. 1616: m.p.98-99 °C. Anal. Calcd. for $C_9H_5O_2Cl$, C, 59.85; H, 2.77; Cl, 19.65. Found, C, 59.74; H, 2.83; Cl, 19.71.

Diphenyl 1-N-benzyloxycarbonylamino-2-phenylethylphosphonate (BL57422) was prepared according to the procedure of Oleksyszyn, J., Subotkowska, L., and Mastalerz, P. (1979) Synthesis 985: m.p. 119-120 °C. Anal. Calcd. for $C_{28}H_{26}O_5NP$: C, 68.94; H, 5.33; N, 2.87. Found: C, 69.00; H, 5.40; N, 2.84.

Diphenyl 1-N-benzyloxycarbonylamino-3-methylthiopropylphosphonate (BL57959) was prepared by the same procedure; m.p. 93-95 °C. Anal. Calcd. for $C_{24}H_{26}O_5NSP$: C, 61.15; H, 5.52; N, 2.97; S, 6.79. Found: C, 61.06; H, 5.60; N, 2.91; S, 6.88.

Diphenyl 1-N-benzyloxycarbonyl-2-methyl-propylphosphonate (BL57968) was prepared by the same procedure; m.p. 104-105 °C. Anal. Calcd. for $C_{24}H_{26}O_5NP$: C,65.56; H, 5.92; N, 3.19. Found: C, 65.67; H, 5.98; N, 3.13.

Diphenyl 1-N-(carbobenzyloxy-phenylalanyl)amino-2-phenylethylphosphonate (BL57842). To a solution of 2.3 g (6.5 mmol) of diphenyl 1-amino-2-phenylethylphosphonate [Oleksyszyn, J., Subotkowska, L., and Mastalerz, P. (1979) Synthesis 985] and Cbz-Phe-OH 1.95 g (6.5 mmol) in 50 mL of dry THF, 1.32 g (6.6 mmol) of DCC (dicyclohexylcarbodiimide) was added. After 2 days DCU was filtered off and solution was washed with water, 2 times with 50 mL of 5% $NaHCO_3$, water, 2 times with 10% citric acid, water and dried over $MgSO_4$. After evaporation of the solvent, the resulting oil was crystallized from THF-hexane to give 3.5 g (85%) of the dipeptide as a white solid; m.p. 153-155 °C. Anal. Calcd. for $C_{37}H_{35}O_6N_2P$: C, 70.03; H, 5.52; N, 4.42. Found: C, 69.93; H, 5.61; N, 4.51.

Isatoic anhydride (BL57646). Isatoic anhydride was purchased from Aldrich and recrystallized from ethanol; m.p. 233 °C (decomp). Anal. Cald. for $C_8H_5O_3N$: C, 58.9; H, 3.07; N, 5.59. Found: C, 58.90; H, 3.07; N, 8.52.

Di(4-isovaleroylphenyl)sulfide (BL57940). A mixture of 10.7 g (80.2 mmol) of $AlCl_3$ in 125 mL of methylene chloride was cooled to -30 °C under an argon atmosphere. Diphenylsulfide 5.0 g (26.8 mmol) was added followed by isovaleroyl chloride 7.19 mL (59.0 mmol). The solution was warmed to 0 °C and stirred 1.5 hr, then allowed to reach room temperature and stirred overnight. The mixture was poured onto a mixture of ice and concentrated HCl (5:1). The organic layer was extracted with the methylene chloride, washed with 1N NaOH and dried over $MgSO_4$. After removing the solvent, the residue was recrystallized from cyclohexane to give the product as a white solid (89%); m.p. 80-81 °C. Anal. Calcd. for $C_{22}H_{26}O_2S$: C, 74.54; H, 7,39; S, 9.04. Found: C, 74.45; H, 7.42; S, 8.97.

2-Benzoylbenzisothiazolinone-1,1-dioxide (BL57977). Tetrabutyl ammonium bromide (0.1 g) was added to a suspension of finely ground sodium saccharin (2.0 g, 9.8 mmol) in 30 mL of benzene. Benzoyl chloride (1.2 mL, 10.3 mmol) was added after azeotropic removal of water from this suspension and the resulting mixture was heated to reflux temperature for 3 h. The reaction mixture was filtered while hot, the solid was washed with hot benzene and the filtrate was cooled to 5 °C while scratching the sides of the flask to induce precipitation of the product. The white solid was collected by filtration and recrystallized from benzene/hexane to yield 1.39 g (50%) of pure product; m.p. 162-163 °C (Lit. 160-163 °C). ^1H NMR ($CDCl_3$) d: 8.14 (d, 1H); 8.01 (t, 2H); 7.93 (m, 1H); 7.78 (d of d, 2H); 7.67 (t, 1H); 7.51 (t, 2H). Anal. Calcd. for $C_{14}H_9NO_4S$: C, 58.53; H, 3.16; N, 4.88. Found: C, 58.66; H, 3.19; N, 4.92.

2-(2-Furoyl)benzisothiazolinone-1,1-dioxide (BL57931). 2-Furoyl chloride (7.4 mL, 75 mmol) was added to a suspension of finely ground sodium saccharin in 40 mL THF and the resulting mixture was heated to reflux temperature for 4 h. The reaction mixture was filtered and the filtrate was concentrated to dryness to give a white solid that was recrystallized from ethyl acetate. The yield of pure product was 4.79 g (35%); m.p. 167-168 °C (Lit. 133-135 °C). ^1H NMR ($CDCl_3$) d: 8.19 (d, 1H); 7.99 (d, 2H); 7.93 (m, 1H); 7.73 (d, 1H); 7.58 (d, 1H); 6.66 (d of d, 1H). Anal. Calcd. for $C_{12}H_7NO_5S$: C, 51.98; H, 2.55; N, 5.05. Found: C, 51.83; H, 2.52; N, 5.07.

2-Phenylacetylbenzisothiazolinone-1,1-dioxide (BL57995). Tetrabutyl ammonium bromide (0.1 g) was added to a suspension of finely ground sodium saccharin (8.0 g, 39 mmol) in 50 mL of benzene. Phenylacetyl chloride (7.7 mL, 58 mmol) was added to this suspension after azeotropic removal of water and the resulting mixture was heated at reflux temperature for 4 h. The reaction mixture was filtered while hot and the solid was washed with hot benzene. Hexane was added to the filtrate and the solid that resulted was filtered and recrystallized from benzene/hexane to yield 5.52 g (47%) of pure product; m.p. 177-179 °C. ^{1}H NMR ($CDCl_3$) d: 8.17 (d of d, 1H); 7.99-7.87 (m, 3H); 7.38-7.28 (m, 5H); 4.39 (s, 2H). Anal. Calcd. for $C_{15}H_{11}NO_4S$: C, 59.79; H, 3.68; N, 4.65. Found: C, 59.71; H, 3.73; N, 4.61.

2-Diphenylacetylbenzisothiazolinone-1,1-dioxide (BL57986). Thionyl chloride (7 mL, 100 mmol) was added to a solution of diphenylacetic acid (10 g, 50 mmol) in 50 mL of benzene and the resulting mixture was heated to reflux temperature for 1 h. Excess thionyl chloride and benzene were removed under reduced pressure (1 mm Hg) and then tetrabutyl ammonium bromide (0.1 g), fresh benzene (50 mL) and finely ground sodium saccharin (6.8 g, 30 mmol) were added in one portion. The resulting mixture was heated to reflux temperature for 4 h and was then filtered while hot. The white solid that was collected was washed with hot benzene and the filtrate was concentrated to a small volume (ca. 10 mL). The white solid that came out of solution was collected by filtration and recrystallized from benzene/hexane to yield 5.43 g (48%) of pure product; m.p. 195-197 °C. ^{1}H NMR ($CDCl_3$) d: 8.08 (d, 1H); 7.95 (d, 2H); 7.89-7.85 (m, 2H); 7.38-7.27 (m, 10H); 6.30 (s, 1H). Anal. Calcd. for $C_{21}H_{15}NO_4S$: C, 66.83; H, 4.01; N, 3.71. Found: C, 66.89; H, 4.01; N, 3.66.

Z-Pro-Phe^P(OPh)$_2$. Z-Pro-OH (1.5 g, 6 mmol) was dissolved in 60 mL of dry THF and cooled to 0 °C. Diphenyl a-amino-2-phenylethylphosphonate (2.1 g, 6 mmol) and DCC (1.22 g, 6 mmol) were added to this solution. The reaction mixture was stirred for 6 h at 0 °C and overnight at room temperature. The precipitated DCU was removed by filtration and 50 mL of ethyl acetate was added to the filtrate. The solution was washed twice with succesive portions of 10% citric acid, water, 4% sodium bicarbonate and water. The resulting solution was dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo* to a residue that was dissolved in 10 mL CH_2Cl_2. The small amount of DCU that came out of solution was removed by filtration and 30 mL of pentane was added to the filtrate to effect crystallization of the product. After a few days the product was filtered and recrystallized from CH_2Cl_2-Hexane to yield 1.86 g (53%)

of pure product as a white solid; m.p.: 81-84 °C. Anal. Calcd. for $C_{33}H_{33}O_6N_2P$: C, 67.81; H, 5.65; N, 4.79. Found: C, 67.56; H, 5.79; N, 4.72.

Suc-Val-Pro-Phe^P(OPh)$_2$ (BL59382). Palladium on charcoal (0.1 g, 5% catalyst) was added to a solution of 1.17 g (2 mmol) of Z-Pro-Phe^P(OPh)$_2$ in 50 mL of methanol and the resulting mixture was stirred under an atmosphere of hydrogen at room temperature for 2 h. The reaction mixture was filtered through Celite and Z-Val-OH (0.5 g, 2 mmol) was added to the filtrate. The solvent was removed *in vacuo* and the residue was dissolved in 60 mL of dry THF. DCC (0.4 g, 2 mmol) was added to this solution and the resulting mixture was kept for 6 h at 0 °C and overnight at room temperature. The resulting DCU was removed by filtration and the filtrate was washed with water, twice with 4% $NaHCO_3$, water and twice with 10% citric acid and water. After drying over $NaSO_4$, the solvent was removed *in vacuo* and the oil that resulted was dried under low pressure for a few hours. The dry semisolid was dissolved in 60 mL of ethyl acetate and succinic anhydride (0.2 g, 2 mmol) was then added. After addition of 0.1 g of 5% Pd/C the resulting mixture was stirred under a hydrogen atmosphere until TLC indicated only one new spot. The catalyst was then removed by filtration through Celite and the filtrate was washed several times with water. The resulting solution was dried over $MgSO_4$, filtered and evaporated to dryness *in vacuo* to yield 0.9 g (65%) of product as a hygroscopic solid; m.p.: 50-53 °C. ^{31}P NMR d: 19.75; 19.23 ppm. Anal. Calcd. for $C_{34}H_{40}O_3N_3P \cdot 2H_2O$: C, 59.56; H, 6.42. Found: C, 59.59; H, 6.42.

2-Cyanomethyl Benzisothiazolinone-1,1-dioxide (BM00464). Sodium saccharin (5.0 g, 24 mmol) was suspended in 50 mL of benzene and tetrabutyl ammonium bromide (20 mg) was then added. Water was removed azeotropically from this mixture by heating the suspension to reflux temperature for 45 min. After removal of water, bromoacetonitrile (1.8 mL, 25.6 mmol) was added and the reaction mixture was heated to reflux temperature for 14 h. The mixture was filtered while hot and the solid was washed with hot benzene (5 x 5 mL). The filtrate was concentrated *in vacuo* to a minimum volume (c.a. 5 mL) and hexane was then added to effect complete precipitation of the product. After one recrystallization from chloroform, the yield of pure product was 2.15 g (39.6%); m.p.: 134-5 °C. ^1H NMR ($CDCl_3$) d: 8.17 (d of d, 1H); 8.02-7.89 (m, 3H); 4.63 (s, 2H). Anal. Calcd. for $C_9H_6N_2O_3S$: C, 48.64; H, 2.72; N, 12.61. Found: C, 48.73; H, 2.76;
N, 12.52.

2-Ethoxy-4H-3,1-benzoxazin-4-one (BM00651). To a solution of anthranilic acid (6.85 g, 0.05 mol) in dry pyridine (50 ml) at room temperature under anhydrous conditions was added ethyl chloroformate (19.2 ml, 0.2 mol) dropwise over a 15 min period. After stirring for 2 h, the solvent was removed under reduced pressure and the residue was stirred vigrously into 100 ml of ice-cold water. The insoluble solid was filtered, washed with water and air-dried. The crude product was dissolved in ethyl acetate, treated with charcoal and recrystallized to give the benzoxazinone as a white solid (8.5 g, 89%); m.p. 88-90° C. Anal. Calcd. for $C_{10}H_9NO_3$: C, 62.83; H, 4.71; N, 7.33. Found: C, 66.78; H, 4.73; N, 7.31.

5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide (BM00491) was purchased from Eastman Kodak; m.p.: 142-144° C. Anal. Calcd. for $C_7H_5O_5NS$; C, 39.04; H, 2.32; N, 6.51; S, 14.87. Found: C, 39.12; H, 2.33; N, 6.51; S, 14.96.

4-Chloro-3-(3-isothioureidopropoxy)isocoumarin (BM00642). This inhibitor was prepared as described by Kam, C-M., Fujikawa, K., and Powers, J. C. (1988) *Biochemistry 27*, 2547; m.p. 174-176° C (decomp). Anal. Calcd. for $C_{13}H_{14}BrClO_3N_2S$; C, 39.65; H, 3.56; N, 7.12; S, 8.13. Found: C,39.70; H, 3.62; N, 7.08; S, 8.23.

3-Benzyloxy-4-chloroisocoumarin (BM00482). This inhibitor was prepared as described by Harper, J. W, and Powers, J. C (1985) *Biochemistry 24*, 7200; m.p. 90-92° C. Anal. Calcd. for $C_{16}H_{11}O_3Cl$; C, 67.02; H, 3.84; Cl, 12.38. Found: C, 66.93; H, 3.92; Cl, 12.28.

4-Chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin (BM01096). To 3.05 g (0.012 mol) of 7-amino-4-chloro-3-propoxyisocoumarin [Harper, J. W., and Powers, J. C. (1985) *Biochemistry 24*, 7200] in 20 ml of dry THF, 1.44 g (0.012 mol) of phenylisocyjanate was added. The solution was kept at room temperature for a few days and the solid which crystallized out was filtered, washed with pentane, and air-dried; yield 3.3 g (74%), m.p. 235-236° C (decomp). Anal. Calcd. for $C_{19}H_{17}ClO_4N_2$; C, 61.22; H, 4.56; N, 7.52; Cl, 9.52. Found: C, 61.30; H, 4.62; N, 7.51; Cl, 9.60.

***p*-Guanidino benzoic acid (BM01363).** This compound was prepared according to the procedure of Chase, T., and Shaw E.(1970) *Methods in Enzymol. 19*, 22; m.p. 274° C (dec.). Anal. Calcd. for $C_8H_{10}ClN3O2$: C, 44.56; H, 4.67; N, 19.49; Cl, 16.44. Found: C, 44.73; H, 4.65; N, 19.34; Cl, 16.58.

Ethyl *p*-guanidino benzoate (BM01185). This compound was made according to the procedure of Beyerman, H. C., and Bontekoe, J. S. (1953) *Rec. Trav. Chim. Pays-Bas 72*, 643; m.p. 164-5° C (dec.). Anal. Calcd. for $C_{10}H_{14}ClN_3O_2$: C, 49.28; H, 5.79; N, 17.24; Cl, 14.55. Found: C, 49.34; H, 5.79; N, 17.17; Cl, 14.52.

O-(*p*-Guanidinobenzoyl)glycolamide Hydrochloride (BM03143). (Ganu and Shaw, 1981) Water was removed azeotropically from a mixture of *p*-guanidinobenzoic acid hydrochloride(2.51 g, 12 mmol) and N-tetrabutylammonium bromide (100 mg) in 50 mL of benzene. 1,2,2,6,6-Pentamethylpiperidine (PMP) was then added (2.1 mL, 12 mmol), followed by 2-chloroacetamide (1.2 g, 13.2 mmol). The resulting mixture was heated to reflux temperature for 24 hours, it was filtered while hot and the beige solid was throroughly washed with acetone. The crude solid was recrystallized from 95% ethanol to yield 1.06 g (34%) of pure product as a white solid, mp 249-51°C. ^{1}H NMR (d_6-Me_2SO) d: 8.05 (d,2H); 7.75 (broad s, 3H); 7.58 (broad s, 1H); 7.36 (d,2H); 7.28 (broad s, 1H); 4.66 (s, 2H). Anal. Calcd. for $C_{10}H_{13}ClN_4O_3$: C, 44.04; H, 4.80; N, 20.55; Cl, 13.00. Found: C, 43.91; H, 4.85; N, 20.45; Cl, 12.93.

N-(*p*-Guanidinobenzoyl)valine Amide Hydrochloride (BM02655). Triethylamine (3.2 mL, 23 mmol) was added to a solution of valine amide hydrochloride (3.1 g, 23 mmol) in 100 mL of DMF. 1-Hydroxybenzotriazole (HOBt, 3.15 g, 23 mmol) was added to this mixture, followed by *p*-guanidinobenzoic acid hydrochloride (5.0 g, 23 mmol) and the reaction mixture was then cooled in an ice bath. N,N-diclyclohexylcarbodiimide (DCC, 5.2 g, 25 mmol) was added to the cold (0-5 °C) solution and the resulting mixture was stirred at 5-10 °C for 24 h. The white solid in suspension (DCU) was filtered and washed with EtOAc. The filtrate was concentrated to dryness *in vacuo* to a foam which was triturated with chloroform (5 x 200 mL) with vigorous mechanical stirring to remove $NEt_3 \cdot HCl$. The resulting amorphous solid was taken up in 100 mL of water, filtered through Celite to remove HOBt and the filtrate was lyophylized to yield 5.13 g (71%) of pure product as a white amorphous solid. ^{1}H NMR (d_6-Me_2SO) d: 10.25 (br.s, 1H); 8.22 (d, 1H), 7.98 (d, 2H); 7.68 (br.s, 3H); 7.54 (s, 1H); 7.30 (d, 2H); 7.08 (br.s, 1H); 4.26 (t, 1H); 2.11 (m, 1H); 0.91 (d, 3H); 0.89 (d,3H). Anal Calcd. for $C_{13}H_{20}ClN_5O_2 \cdot 0.8\ H_2O$: C, 47.56; H, 6.63; N, 21.33; Cl, 10.80. Found: C, 47.56; H, 6.52; N, 21.26; Cl, 10.68.

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Annual Report

PROTEASE INHIBITORS AS ANTIVESICANTS

JAMES C. POWERS

School of Chemistry
Georgia Institute of Technology
Atlanta, GA 30332

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19. ABSTRACT

Sulfur mustard is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis. The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister. A total of 39 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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In conducting research utilizing recombinant DNA technology, the investigator adhered to current guidelines promulgated by the National Institutes of Health.

2/25/1991

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PROTEASE INHIBITORS AS ANTIVESICANTS

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ABSTRACT

Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic (protein degrading) enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis (outer non-vascular layer of skin). The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister.

A total of 39 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 5 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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BACKGROUND

Sulfur Mustard. Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. Antimustard ointments which attempt to inactivate the free mustard are ineffective since the mustard quickly reacts with components in the skin and other tissues. Therefore, an effective therapy for sulfur mustard exposure must be based on reversing the physiological processes that result upon contact with this potent vesicant (Cullumbine, 1947).

Bis-(2-chloroethyl)sulfide is a potent alkylating agent which can react with the heterocyclic bases in nucleic acids (Ludlum et al., 1984) and with a wide variety of the side chain functional groups (thiols, thioethers, carboxyl groups, amino groups, imidazole rings, etc.) found in proteins. The majority of the sulfur mustard is secreted in the form of various metabolites such as thiodiglycol, but some is carried by the circulation to other organs, and a significant portion is stored in skin reservoirs (Klain and Bonner, 1987). While the most significant mustard induced injury occurs in the skin (vesication and inflammation), significant numbers of mustard casualties have ocular injuries and cornea impairment for 2-4 months. In cases of severe exposure, there is serious lung and bone marrow damage which results in death.

Mechanism of Sulfur Mustard Induced Blistering. The molecular mechanisms by which sulfur mustard causes toxicity are unknown but mustard is a powerful alkylating agent of DNA and RNA. Papirmeister has suggested that the alkylated purine bases in DNA are unstable and undergo both spontaneous and enzymatic depurination (Papirmeister et al., 1985). This results in DNA strand breaks, and activation of nucleases and other DNA repair mechanisms. As a result, poly(ADP-ribose)polymerase is activated, NAD^+ is depleted, glycolysis is inhibited, and the hexose monophosphate shunt is stimulated (Meier et al., 1987). This causes the release of potent proteolytic enzymes which produces the observed pathology of basal cell necrosis and vesication.

Evidence for the Papirmeister hypothesis includes the isolation and structural characterization of several DNA alkylation products upon treatment of DNA with sulfur mustard (Benschop et al., 1989) and the demonstration of single strand breaks in the DNA after exposure of keratinocyte cultures to low levels of sulfur mustard (Bernstein et al., 1989). In addition, other agents which result in DNA damage such as UV light and radiation have been shown to stimulate the synthesis or release of proteases in fibroblast cultures (Miskin and Reich, 1980).

Proteases are normally controlled by natural plasma protein protease inhibitors such as α_1-protease inhibitor, α_1-

antichymotrypsin, and α_2-macroglobulin. If this antiprotease screen is destroyed, tissue destruction results. Several of the plasma serpins (serine protease inhibitors) including α_1-protease inhibitor and α_1-antichymotrypsin have essential methionine residues and are susceptible to inactivation by oxidizing agents or alkylating agents. A single dose of sulfur mustard in the mouse brain has recently been shown to cause a burst of oxidants (Elsayed et al., 1989). The serpin screen could then be removed directly by sulfur mustard alkylation or indirectly by oxidation as a result of this oxidative burst. Thus, sulfur mustard exposure probably results both in the release of powerful proteolytic enzymes and in the partial destruction of the protease inhibitor screen which would normally protect the organism from proteolysis.

Other Blistering Disease States Involve Proteases. Blistering disease states which have been described include dermatitis herpetiformis (DH), bullous pemphigoid (BP), chronic bullous disease of childhood, and pemphigus vulgaris. These diseases are characterized by destruction of various connective tissue components of the epidermis or dermis followed by tissue separation and the formation of fluid-filled blisters. Blister fluids from patients with all of these diseases have been shown to contain proteases including elastase and collagenase (Oikarinen et al., 1983). Human polymorphonuclear leukocyte elastase is the major enzyme in DH fluid, while BP fluid predominantly contains the metalloprotease collagenase. A trypsin-like enzyme and a thiol protease have also been implicated in blister formation respectively in recessive dystrophic epidermolysis bullosa and epidermolysis bullosa simplex (Takamori et al., 1985). Incubation of normal human skin with the blister fluid from patients with epidermolysis bullosa letalis, a severe and usually fatal congenital blister disease, results in dermal-epidermal separation. A number of common serine protease inhibitors prevented the separation (Matsumoto and Hashimoto, 1986).

Proteases are Associated with Inflammation. Proteases are important mediators and modulators of inflammation and have been demonstrated in non-blistering inflammatory disease states such as psoriasis and arthritis. The most abundant enzymes are the serine proteases elastase and cathepsin G (a chymotrypsin-like enzyme) from leukocytes; chymases (chymotrypsin-like enzymes), and tryptases (trypsin-like) enzymes from mast cells; plasminogen activator; and the metalloprotease collagenase from leukocytes. These enzymes are capable of cleaving a variety of connective tissue proteins including elastin, collagen, proteoglycans, and other basement membrane components.

Sulfur Mustard Induced Inflammatory Lesions Contain Proteases and Protease-Inhibitor Complexes. The proteolytic enzymes released upon exposure to sulfur mustard have not yet been isolated or characterized, but likely candidates include chymases and tryptases from mast cells, elastase and cathepsin G from leukocytes, plasminogen activator, and collagenase. Culture

fluids from mustard-induced inflammatory lesions in rabbit skin show 3 to 6 fold increased levels of proteases both in developing and healing lesions (Higuchi et al., 1987). These fluids will hydrolyze two synthetic peptide substrates, Boc-Leu-Gly-Arg-AFC (Boc = *t*-butyloxycarbonyl, AFC = 7-amino-4-trifluoromethyl coumarin) and Bz-Phe-β-naphthyl ester (Bz = benzoyl). The first peptide is a substrate for trypsin, tryptases, plasmin, plasminogen activator and other trypsin-like enzymes, while the latter is a substrate for chymotrypsin-like enzymes including chymases and cathepsin G. The rabbit skin culture fluids did not consistently hydrolyze four other synthetic peptide substrates (two for elastase and two for cathepsin G) or the protein elastin (elastase's natural substrate). Exposure of human skin in culture to sulfur mustard results in a 41 % increase in plasminogen activator activity (Dannenberg et al., 1989), an enzyme which is known to be associated with blister formation (Hashimoto et al., 1983). The enzymatic activity of chymases, tryptases, and angiotensin converting enzyme toward small synthetic substrates were not elevated.

The proteases found in the culture fluids from mustard-induced inflammatory lesions in rabbit skin are not present as free active enzymes, but are found as inactive complexes with their natural plasma protease inhibitors α_1-protease inhibitor and α_2-macroglobulin (Harada et al., 1987; Dannenberg et al., 1987; Higuchi et al., 1987). These complexes are formed as natural protein protease inhibitors from the plasma react with the proteases being released at the site of inflammation. The protease-inhibitor complexes are incapable of hydrolyzing protein substrates and complex formation thus protects the organism from further damage. The natural plasma protease inhibitors are probably not completely destroyed by exposure to low levels of sulfur mustard and are still available to react with some of the proteases released in the blister.

Protease-inhibitor complex formation hinders the identification of the proteases present in sulfur mustard induced culture fluid. Complexes of elastase and cathepsin G respectively with α_1-protease inhibitor and α_1-antichymotrypsin are inactive toward both synthetic peptide substrates and natural protein substrates. Thus the lack of hydrolysis of elastin or elastase substrates does not preclude the presence of inactivated elastase in the culture fluids. Similarly the lack of hydrolysis of the protein fibrin by the culture fluids doesn't exclude the presence of plasmin or plasminogen activator. In summary, it is now clear that there is a chymotrypsin-like enzyme (chymase), a trypsin-like enzyme (tryptase), and plasminogen activator in the sulfur mustard induced inflammatory lesions, but the presence of other enzymes has not been excluded.

Skin Serine Proteases Have Been Isolated and Characterized. The dermis of human skin is a rich source of mast cells and salt extraction of human skin has yielded two serine proteases, a chymase and a tryptase. These serine

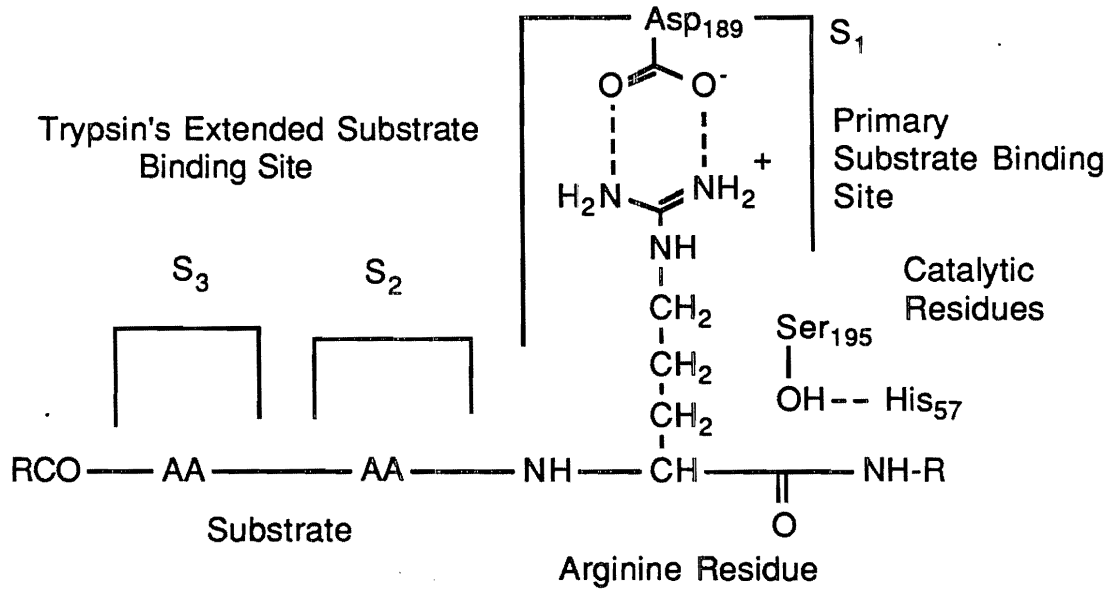
proteases are localized in the granule fraction of mast cells, a cell type which is located predominantly in connective tissue. The chymase has been demonstrated immunocytochemically to bind to the dermo-epidermal junction in skin (Sayama et al., 1987). Both the mast cell chymase and tryptase are able to specifically cleave proteins found in the dermal-epidermal boundary and cause vesication. The chymase is incompletely inhibited by plasma due to a 650 fold slower rate of reaction with the serpins α_1-protease inhibitor and α_1-antichymotrypsin (Schechter et al., 1989), while the tryptase appears not to be inhibited by most protein protease inhibitors (Schechter et al., 1983). This may explain the ready detection of chymase and tryptase activity in culture fluids from mustard-induced lesions in rabbit skin.

Rat mast cells contain two chymases (RMCP I and RMCP II) which have been more extensively characterized than the chymase from human skin. Both of these serine proteases are highly homologous to human mast chymase and human leukocyte cathepsin G. RMCP I and RMCP II have both been sequenced and the x-ray crystal structure of RMCP II has been determined, while the x-ray structure of RMCP I is underway (Woodbury and Neurath, 1980; Remington et al., 1988). The sequence of dog mast cell tryptase and a related dog mast cell protease have been determined by gene sequencing techniques (Vanderslice et al., 1989) and it is likely that the sequences for human skin tryptase and chymase will be available in the next few years.

The substrate specificity and inhibition profile of human skin chymase, human skin tryptase, RMCP I, RMCP II, and related enzymes have been studied in the laboratory of the principle investigator (Powers et al., 1985). Human skin chymase hydrolyzes peptide substrates containing aromatic amino acid residues and prefers Phe-AA and Tyr-AA bonds over Trp-AA bonds (AA = any amino acid residue) in contrast to chymotrypsin which prefers Trp over Phe and Tyr. One of the best peptide substrates is Suc-Phe-Val-Pro-Phe-NA (Suc = succinyl, NA = 4-nitroanilide). Human skin tryptase is a trypsin-like enzyme, but seems to prefer double basic residues in its substrates (Tanaka et al., 1983). For example, the thioester substrate Z-Lys-Arg-SBu-i (Z = benzyloxycarbonyl, SBu-i = thioisobutyl ester) is hydrolyzed by human skin tryptase with a k_{cat}/K_M = 59,000,000 $M^{-1}s^{-1}$, a second order rate constant which is close to the diffusion controlled rate.

Serine Protease Specificity. The specificity of serine proteases toward natural peptide substrates or synthetic inhibitors is determined by the nature of the primary substrate specificity pocket (S_1) and secondary subsites (S_2, S_3, etc.) on the surface of each individual enzyme. Trypsin's primary specificity site contains an Asp residue in the back of the S_1 pocket so that trypsin will only bind to and hydrolyze peptide substrates containing lysine or arginine residues (a schematic model of trypsin with a bound substrate is shown below). The

three-dimensional structure of chymotrypsin is quite similar except that the Asp-189 in trypsin is replaced by Gly-189 in chymotrypsin. As a result the S_1 pocket of chymotrypsin in very hydrophobic and chymotrypsin prefers substrates containing aromatic amino acid residues such as Trp, Tyr, and Phe. With many serine proteases, interactions of inhibitors with the extended substrate binding site (S_2, S_3, etc.) are important to increase the specificity and reactivity of the inhibitor. This is clearly the case with human skin chymase and tryptase. For example, interaction of the Lys in the substrate Z-Lys-Arg-SBu-i with the S_2 subsite of human tryptase results in an accelerated rate of hydrolysis, while little change in hydrolysis rate is observed with trypsin.



HYPOTHESIS

It is clear—no matter the exact mechanism of their release or their source—that proteases are major factors in the tissue destruction that accompanies mustard induced vesication. We propose that protease inhibitors will be effective antivesicants and should be useful both in preventing blistering and in the treatment of blisters. Appropriate target proteases are the mast cell chymase and tryptase, serine proteases which are localized in the skin and have the ability to cleave proteins at the dermal-epidermal junction. However other serine protease such as elastase and cathepsin G from leukocytes, and plasminogen activator may also be involved. Evidence for the involvement of other classes of proteases such as the metalloprotease collagenase or the thiolprotease cathepsin B is incomplete or lacking at present, although the mast cell tryptase is able to activate latent collagenase (Gruber et al., 1989).

Research Strategy. Since the exact target enzyme (or enzymes) is not known with certainty, we have decided to synthesize general serine protease inhibitors, specific chymase inhibitors, specific tryptase inhibitors, specific plasminogen activator inhibitors, and specific inhibitors for other enzymes as appropriate. We plan to shift our emphasis to a particular enzyme or group of enzymes when more biological data is obtained on the role of specific proteases in blistering or when we receive animal test data on the compounds which we have already submitted. Until that time, we plan to submit for testing a wide variety of potentially active structures including both general serine protease inhibitors and inhibitors which are more enzyme specific. During the last portion of this contract, we have been focusing on inhibitors for tryptase since this may be the most significant protease involved in blistering.

PROGRESS REPORT

Research Goals

1. Prepare and submit for animal testing 3-5 inhibitors of serine proteases such as 3,4-dichloroisocoumarin and saccharins each year.

2. Prepare and submit for testing 3-5 inhibitors of human skin chymase each year.

3. Prepare and submit for testing 3-5 inhibitors of human skin tryptase each year.

4. Prepare and submit for testing each year 3-5 inhibitors for other human serine proteases-such as human leukocyte elastase, cathepsin G, and plasminogen activator-which may have a role in vesication.

5. Assay all inhibitors with human skin serine proteases and related enzymes for *in vitro* effectiveness.

Research Progress-Summary

1. A total of 39 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing during the first two years of this contract. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 5 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. All the inhibitors submitted are listed in the following table along with their sample numbers.

2. Various inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

SAMPLES SUBMITTED: DMAD17-89-C-9008

BOT-NUM	WR-NUM	SUB-ID	SUB	RCHD	COMPOUND
ISOCOUMARINS (9 submitted)					
BL58572	268195	II-148		89/05/15	3,4-dichloroisocoumarin
BL57637	268119	II-134		89/03/20	3-chloroisocoumarin
BL57413	259666	II-137		89/03/13	4-chloro-3(2-phenylethoxy)isocoumarin
BM00482	268387	II-151		89/08/08	4-chloro-3-benzyloxyisocoumarin
BM04319	268693	MA-134		90/04/17	7-amino-4-chloro-3-cyclohexylmethoxyisocoumarin
BM04668	268715	GP1		90/05/17	4-chloro-3-methylisocoumarin
BM01096	268440	JO-138		89/09/12	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxy isocoumarin
BM00642	268397	II-152		89/08/21	4-chloro-3-(3-S-isothiureidopropoxy)isocoumarin
BM06288		GP2		90/09/05	4-chloro-3-methoxyisocoumarin
SACCHARINS (5 submitted)					
BL57977	268145	MA-84		89/04/19	N-benzoyl saccharin
BL57995	268147	MA-89		89/04/19	N-phenylacetyl saccharin
BL57986	268146	MA-87		89/04/19	N-diphenylacetyl saccharin
BL57931	268141	MA-86		89/04/19	N-furoyl saccharin
BM00464	268385	MA-96		89/08/01	N-cyanomethyl saccharin
PHOSPHONATES (6 submitted)					
BL57959	268143	II-22		89/04/19	Z-Met^{P}(OPh)$_2$
BL57968	268144	II-138A		89/04/19	Z-Val^{P}(OPh)$_2$
BL57422	259858	II-137A		89/03/13	Z-Phe^{P}(OPh)$_2$
BL57842	268132	II-139		89/04/10	Z-Phe-Phe^{P}(OPh)$_2$
BL59382	268241	II-147		89/06/14	Suc-Val-Pro-Phe^{P}(OPh)$_2$
BM04328	268694	III-3		90/04/17	Z-NHCH(AmPh)PO(OPh)$_2$

p-GUANIDINOBENZOIC ACID DERIVATIVES (5 submitted)

BM01363	268476	MA101		89/10/06	p-guanidino benzoic acid
BM01185	184335	MA-111		89/09/19	ethyl-p-guanidinobenzoate
BM02655	268570	MA-121		89/12/13	N(p-guanidinobenzoyl)valine amide
BM03143	268596	MA-115		90/01/23	O(p-guanidinobenzoyl)glycol amide
BM06304		MA-148		90/09/11	4-cyanophenyl p-guanidinobenzoate

BENZOXAZINONES (3 submitted)

BM00651	268398	JO-12		89/08/21	2-ethoxybenzoxazinone
BM05441		GP3		90/06/29	2-phenylamino-4H-3,1-benzoxazin-4-one
BM05807		GP4		90/07/24	2-benzylamino-4H-3,1-benzoxazin-4-one

BENZAMIDINES (5 submitted)

BM06804		GP5		90/11/02	1-(4-amidinophenyl)-3-phenylurea
BM06840		GP6		90/11/14	1-(4-amidinophenyl)-3-(4-chlorophenyl)urea
BM07481		GP7		90/12/18	1-(4-amidinophenyl)-3-benzylurea
BM07829		GP8		91/01/31	1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea
		GP9	91/02/11		(4-amidinobenzyl)benzyl ether

MISC. (6 submitted)

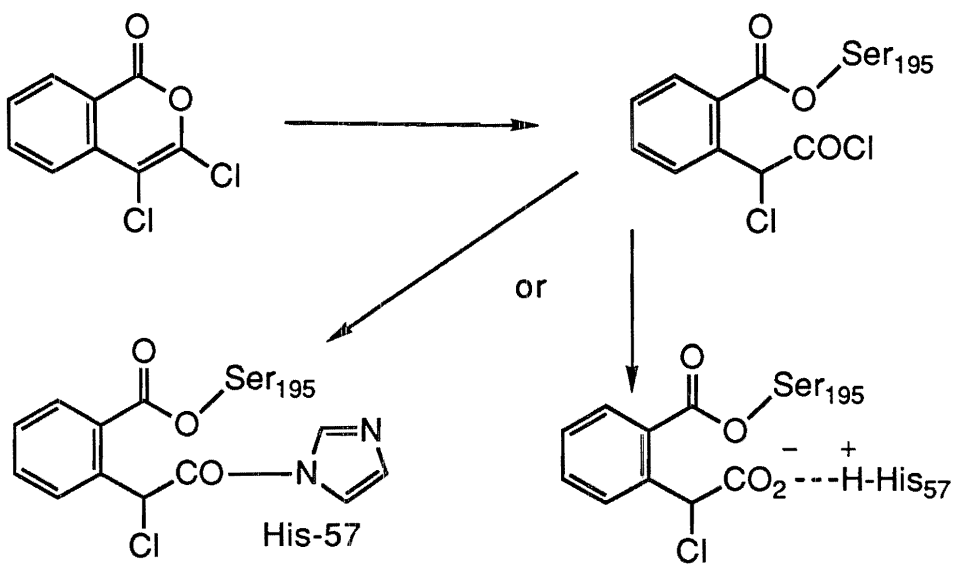
BL57646	015392	II-137B		89/03/20	isatoic anhydride
BL57940	268142	II-145		89/04/19	di(4-isovaleroylphenyl)sulfide
BM00491	099874	II		89/08/08	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide
BM07490		MA-60		90/12/18	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole
BM07838		MA-76		91/01/31	1-benzoyloxy-1,2,3-benzotriazole
BM07132		MA-68		90/11/27	N-(2-isothiureidoethyl)pthalimide

TOTAL SUBMITTED (2/14/91): 39

Inhibitors Submitted.

Isocoumarins-General Inhibitors. Dichloroisocoumarin (WR268195) is an excellent general inhibitor of serine proteases and was discovered in the laboratory of the principal investigator (Harper et al., 1985). With the exception of the bacterial enzyme subtilisin, 3,4-dichloroisocoumarin is an inactivator of all serine proteases which have been tested, including human leukocyte elastase, human skin chymase, dog skin chymase, rat mast cell protease I, and rat mast cell protease II.

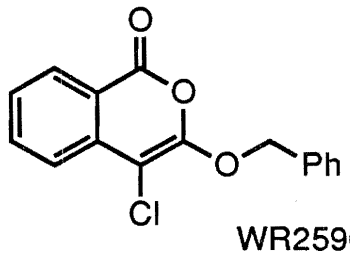
The dichloroisocoumarin ring system contains a masked acid chloride (or ketene) functional group which is exposed when an acyl enzyme is formed upon reaction with the active site serine of a serine protease (Harper et al., 1985). The acyl enzyme (top right of figure) which is formed initially can react further by acylating the active site histidine to form a doubly acylated enzyme derivative (bottom left) or can hydrolyze to form an acyl enzyme stabilized by a salt link between the protonated histidine and the inhibitor carboxyl group (bottom right). The monochloro derivative, 3-chloroisocoumarin (WR268119), inhibits chymotrypsin-like enzymes at slower rates than 3,4-dichloroisocoumarin and does not touch trypsin. The acyl enzymes formed upon reaction with dichloroisocoumarin have variable stabilities, but in general the half-lives for reactivation (deacylation) are greater than 8 hrs at pH 7.5.



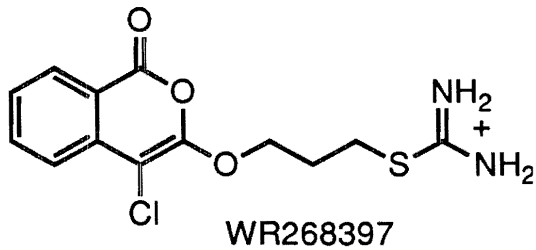
Dichloroisocoumarin and 3-chloroisocoumarin are formed by reaction of homophthalic acid with PCl_5.

Isocoumarins-Specific Inhibitors. We have also synthesized a number of isocoumarin inhibitors which are more specific for the active sites of chymases or tryptases.

Inhibitors targeted for chymase should contain an aromatic side chain which resembles the side chain of Phe, Tyr or Trp, while those inhibitors targeted for tryptase should containing a charged group which resembles the side chain of Arg or Lys. Several of the more specific isocoumarin inhibitors are shown below. Inhibitors with the benzyloxy (such as WR268387) or phenylethoxy groups (WR259666) were targeted at the chymases, while those with basic side chains (such as WR268397) were targeted at the tryptases.

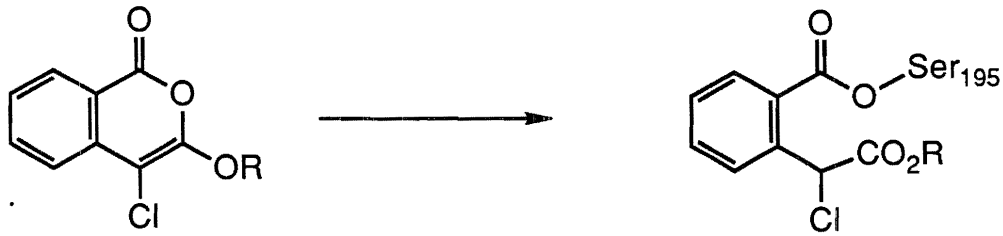


WR259666

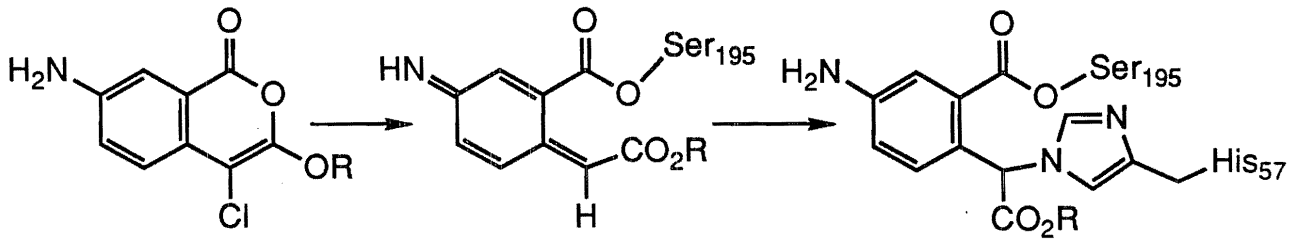


WR268397

The mechanism of inhibition of serine proteases by 3-alkoxy-4-chloroisocoumarins involves acylation of the active site serine-195 to form acyl enzymes with varying stabilities ($t_{1/2}$ = hrs to days) depending on the nature of the alkoxy group (Harper and Powers, 1985).

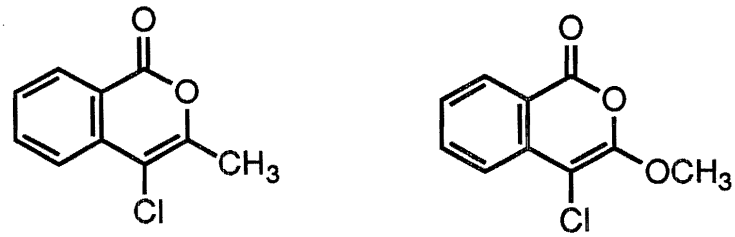


Analogous isocoumarins with electron donating substituents in the 7-position such as 3-alkoxy-7-amino-4-chloroisocoumarins (below) are mechanism-based or suicide inhibitors of serine proteases (Powers et al., 1989). These inhibitors also acylate serine proteases, but form stable acyl enzymes which are not reactivated upon long standing or upon treatment with hydroxylamine. The inhibition mechanism involves formation of an acyl enzyme which can then eliminate chloride to form a quinone imine methide (center). This intermediate then irreversibly alkylates His-57 with the formation of a stable covalent bond between enzyme and inhibitor. This mechanism is supported by x-ray crystallographic studies of complexes of isocoumarin inhibitors bound to the active site of porcine pancreatic elastase (Bode et al., 1989). Thus far, five separate isocoumarins have been studied crystallographically, two give simple acyl enzyme structures (above), two give acyl enzyme structures where His-57 has been alkylated, and one gives a mixture of both structures.

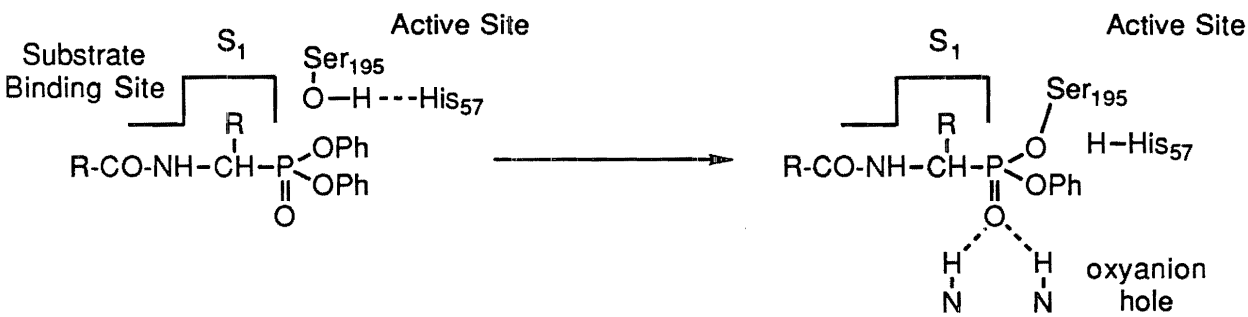


Isocoumarins which have been submitted and should inhibit serine proteases by the above mechanism include 7-amino-4-chloro-3-(cyclohexylmethoxy)isocoumarin (WR268693) and 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin (WR268440).

Two analogs of 3,4-dichloroisocoumarin are shown below. These two compounds were synthesized with the expectation that any isocoumarins with two small electronegative functional groups would be effective general serine protease inhibitors.

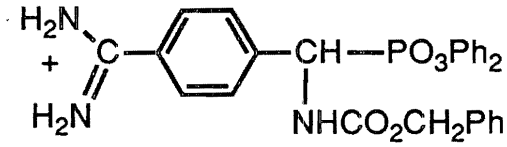


α-Aminoalkylphosphonates Diphenyl Esters. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations (Oleksyszyn and Powers, 1989; 1991). These peptide derivatives phosphonylate the active site serine to form stable phosphonyl derivatives. Good interactions with the S_1 pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative.

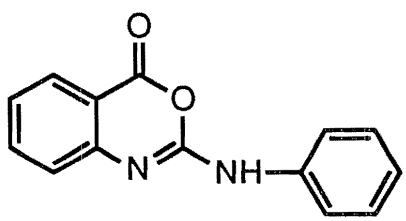


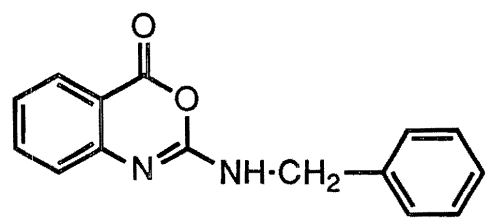
Phosphonate diphenyl ester inhibitors are chemically stable, relatively easy to synthesize, do not react with acetylcholinesterase, form very stable derivatives possibly due to their resemblance to the tetrahedral intermediate involved in peptide bond hydrolysis, and have considerable potential utility as therapeutic agents.

We have submitted a number of simple amino acid and peptide derivatives of phosphonate diphenyl esters including Z-Met^{P}(OPh)$_2$ (WR268143), Z-Val^{P}(OPh)$_2$ (WR268144), Z-Phe^{P}(OPh)$_2$ (WR259858), Z-Phe-Phe^{P}(OPh)$_2$ (WR268132), and Suc-Val-Pro-Phe^{P}(OPh)$_2$ (WR268241). More recently, we have accomplished the synthesis of the amidinophenyl phosphonate derivative (WR268694) shown below. The benzyloxycarbonyl (Z) derivative has been submitted recently for testing. We have also synthesized a few peptide derivatives of the amidinophenyl phosphonate, but not in sufficient quantities for submission.

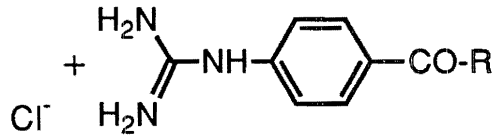


Benzoxazin-4-ones. Substituted benzoxazin-4-ones were discovered to be potent inhibitors of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, cathepsin G, and chymotrypsin by the PI (Teshima et al., 1982). Mechanistic studies by Abeles showed that these compounds were forming stable acyl enzyme derivatives (shown below) with chymotrypsin (Hedstrom et al., 1984) and this has been confirmed by x-ray crystallographic studies with two benzoxazinones bound to PP elastase (Radhakrishnan et al., 1987). Due to the potential of benzoxazinone inhibitors of HL elastase for treatment of emphysema, a group at Syntex Canada has synthesized over 100 new benzoxazinones, carried out a structure-function study as substituents were varied on the ring system, and studied the plasma stability of these compounds (Spencer et al., 1986; Krantz et al., 1987; Krantz et al., 1990). This year we have submitted two benzoxazinones (BM05441 and BM05807) as elastase inhibitors.



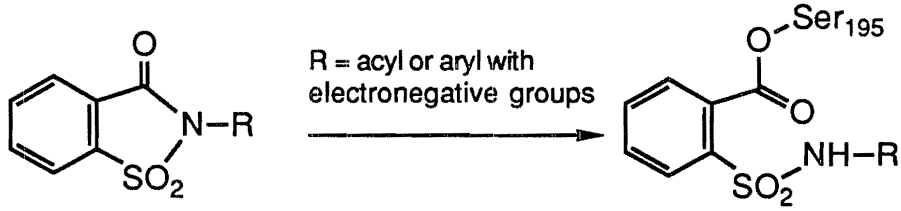


Guanidinobenzoic Acid Inhibitors. Esters of *p*-guanidinobenzoic acid have been reported to be potent inhibitors for various trypsin-like enzymes (Okutome et al., 1984; Fujii et al., 1977) and *p'*-nitrophenyl-*p*-guanidinobenzoate is widely used as an active-site titrant for these enzymes (Chase and Shaw, 1970). An active-site titrant for trypsin-like enzymes developed in the laboratories of the PI is benzyl *p*-guanidinothiobenzoate (Cook and Powers, 1983). We have submitted samples of five *p*-guanidinobenzoic acid derivatives for testing.

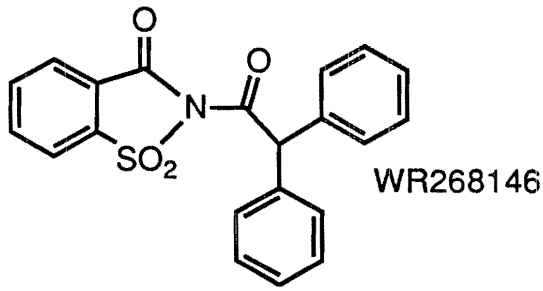


R = OH (WR268476)
OEt (WR184335)
NH-Val-NH_2 (WR268570)
OCH_2CONH_2 (WR268596)
C_6H_4-CN (BM06304)

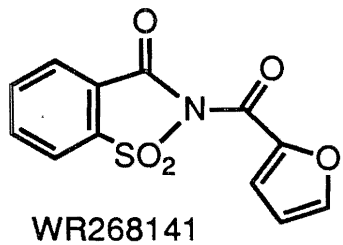
Saccharin Inhibitors. N-Acyl and N-aryl saccharins are potent acylating agents of HL elastase, cathepsin G, and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981). A few of the N-acyl derivatives such as N-furoyl, N-thienoyl, and N-benzoylsaccharin inhibit trypsin with IC_{50} values of 0.7-2.4 μM. These structures were initially designed as acyl transfer reagents, but studies using ^{35}S-labeled N-furoylsaccharin indicated that the saccharin portion of the inhibitor becomes covalently and stoichiometrically bound to both HL elastase and pancreatic elastase upon acylation.



Two of the saccharins which we have submitted are shown below.

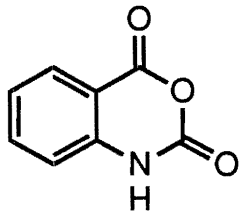


WR268146

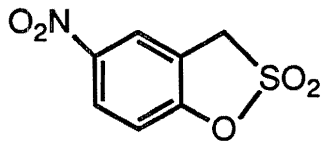


WR268141

Miscellaneous Inhibitors. Two heterocyclic general serine protease inhibitors have been submitted. They are isatoic anhydride which has been shown to acylate the active site of chymotrypsin and form a stable acyl enzyme (Moorman and Abeles, 1982) and 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide which forms a stable sulfonyl derivative also with chymotrypsin.

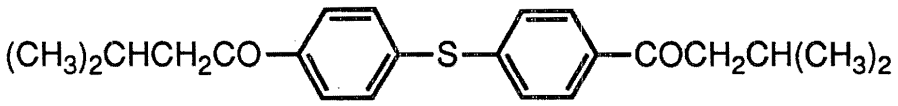


Isatoic Anhydride
WR015392



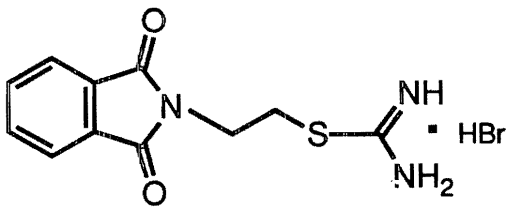
5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide
WR099874

The sulfide shown below is an effective inhibitor of elastase which was discovered in the laboratory of the PI.

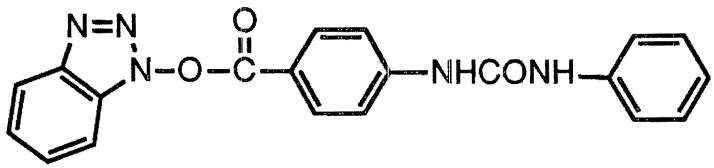


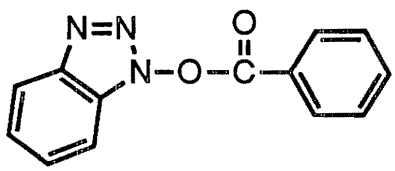
WR268142

The following phthalimide derivative was designed as a trypsin inhibitor.

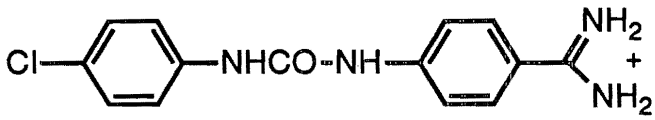


The following acyl derivatives of N-hydroxybenzotriazole inhibit trypsin.

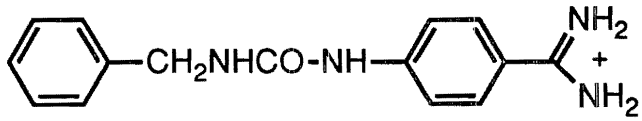




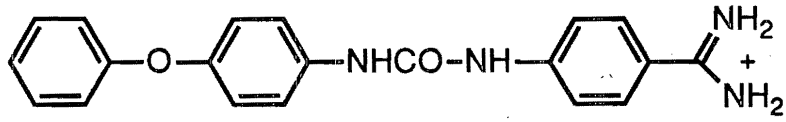
Benzamidine Inhibitors. Five benzamidine inhibitors have been submitted as reversible inhibitors for trypsin-like enzymes. These compounds interact with the S_1 pocket as well as other portions of the active site.



BM06840



BM07481



GP8

Biological Test Data.

All the inhibitors which we have synthesized have been tested for inhibitory potency against a variety of serine proteases. Kinetic data obtained with the various inhibitors are shown in Tables I-III. Most of the inhibitors are irreversible or slowly reversible inhibitors and we report the second order inhibition rate constants $k_{obs}/[I]$. Several of the inhibitors reported in the tables have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. A second order inhibition rate constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing the enzyme and inhibitor in the assays). The half-life of the inhibition reaction can be calculated from the equation $t_{1/2} = 0.693/\{[I]*k_{obs}/[I]\}$. Thus, an inhibitor with a $k_{obs}/[I]$ value of 100,000 $M^{-1}s^{-1}$ would have an inhibition half-life of 6.93 sec. at an inhibitor concentration of 1 μM, while an inhibitor with $k_{obs}/[I]$ = 10,000 would have a half-life of 69 sec. For the few reversible inhibitors investigated, K_I values (dissociation constant of the enzyme-inhibitor complex) or IC_{50} values are given.

The data with bovine chymotrypsin, cathepsin G, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The best isocoumarin inhibitor in this table is 3-benzyloxy-4-chloroisocoumarin (WR268387) with a $k_{obsd}/[I]$ = 12,000 $M^{-1}s^{-1}$ for the human skin chymase. Increasing the length of the side chain at position 3 by one methylene group [4-chloro-3-(2-phenylethoxy)isocoumarin, WR259666] reduces the activity by a factor of 35.

The best phosphonate inhibitor for chymotrypsin-like enzymes is Suc-Val-Pro-Phe^{P}(OPh)$_2$, which corresponds to the sequence of an excellent 4-nitroanilide substrate for these enzymes. NMR studies with chymotrypsin indicate that only one of the two stereoisomers reacts with the enzyme ($k_{obsd}/[I]$ = 146,000 $M^{-1}s^{-1}$ calculated for the single isomer, the value in the table is for the DL mixture). The ^{31}P NMR of chymotrypsin inhibited by this peptide phosphonate shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonylated enzyme derivative (Oleksyszyn and Powers, 1991). The tripeptide phosphonate Suc-Val-Pro-Phe^{P}(OPh)$_2$ makes better interactions with the extended substrate binding site of the enzyme than is possible with the shorter dipeptide or amino acid phosphonate derivatives.

All of the saccharins submitted so far have high $k_{obsd}/[I]$ values with the various chymotrypsin-like enzymes and low IC_{50} values with the elastases tested. One of the better inhibitors in this family is N-furoylsaccharin. The acyl enzymes formed upon acylation of serine proteases by acyl saccharins have variable stabilities. Furoyl saccharin and benzoyl saccharin form inhibited elastase derivatives which are very stable and have half-lives for deacylation of 80-160 hrs. In contrast the

chymotrypsin derivatives have much shorter half-lives in the range of 1.9 hrs. One disadvantage of some acyl saccharins is their fairly rapid hydrolysis at neutral pH values.

The data with porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE) is given in Table II. All the isocoumarin derivatives reported in this table are excellent inhibitors of HLE, the best one being the 7-ureido derivative WR268440 with a $k_{obsd}/[I]$ = 140,000 $M^{-1}s^{-1}$ for HLE. It is also highly selective compared with 3,4-dichloroisocoumarin and 3-chloroisocoumarin which is a logical consequence of the improvement in binding resulting from substitution at the 7-amino group. We have also submitted an excellent benzoxazinone inhibitor for HLE (WR268398). Two analogs of this compound were poor inhibitors of the elastases, and were only moderate inhibitors of chymotrypsin-like enzymes.

The phosphonate derivatives which we have submitted thus far are not good inhibitors for PPE or HLE. This is not surprising since the sequence of the tripeptide was chosen for chymase inhibition and this sequence is very specific for the chymases as discussed earlier. An excellent phosphonate inhibitor for the elastases has been synthesized only in a small scale. This inhibitor, Boc-Val-Pro-Val^{P}(OPh)$_2$, has $k_{obsd}/[I]$ value of 27,000 $M^{-1}s^{-1}$ for human leukocyte elastase (HLE) and 11,000 $M^{-1}s^{-1}$ for porcine pancreatic elastase (PPE). Again this sequence corresponds to a good HLE substrate sequence and at low concentrations this peptide did not react with chymotrypsin.

The inhibition data obtained with bovine trypsin, human lung tryptase, rat skin tryptase, human skin tryptase, and human recombinant tissue plasminogen activator is given in Table III. The best tryptase inhibitor submitted is the 3-(isothioureidopropoxy)isocoumarin WR268397 with a $k_{obs}/[I]$ = 650,000 $M^{-1}s^{-1}$ for the rat skin tryptase, an extremely rapid inhibition rate. However, the acyl-enzyme formed with this inhibitor is unstable and the enzyme regains its activity within 5 min.

Several *p*-guanidinobenzoic acid derivatives have been tested as inhibitors for the various tryptases. One of these derivatives, O-(*p*-guanidinobenzoyl)glycolamide is an excellent inhibitor of the rat skin tryptase, but is a much poorer inhibitor of human skin tryptase. In contrast to the isocoumarin WR268397, the inhibited derivative did not regain enzyme activity upon standing. We pursued this lead and synthesized additional derivatives. The 4-cyanophenyl *p*-guanidinobenzoate was also a very potent inhibitor of trypsin and human lung tryptase. However it is probably not very stable in solution.

We then shifted our emphasis away from guanidino compounds to amidines when we learned that amidines are likely to be less toxic than guanidino derivatives. In the last few months of this contract we have been concentrating only on inhibitors for

tryptases since this may be the more important enzyme in blister formation. Aromatic benzamidine derivatives are reversible inhibitors for trypsin-like enzymes and we have been preparing a series of derivatives to see if we can increase their inhibitory potency for tryptases. The most potent inhibitor thus far for both trypsin and the human lung tryptase is 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea (GP8) which has a K_I value of 1.6 μM with bovine trypsin and inhibits 71% of the activity of human lung tryptase at 226 μM.

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Isocoumarin Inhibitors				
268195	3,4-dichloroIC[g]	570	28	580	27	82
268119	3-chloroIC[g]	330	24	85		
259666	4-chloro-3-(2-phenylethoxy)IC	3800	100	200	340	
268387	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	39,000
268693	7-amino-4-chloro-3-cyclohexylmethoxyIC				25	
268715	4-chloro-3-methyl IC	66	8			
BM06288	4-chloro-3-methoxy IC	206	25			
		Benzoxazinone Inhibitors				
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	77	reactivates			

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI			
	Miscellaneous Inhibitors					
015392	isatoic anhydride	580	114	250		
268142	di(4-isovaleroylphenyl)sulfide		NI[h]	25%[i]		
	Phosphonate Inhibitors					
268143	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		
268144	Z-Val^{P}(OPh)$_2$	0.4	NI[h]	NI[h]		
259858	Z-Phe^{P}(OPh)$_2$	260	76	89		
268132	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI[h]	18		
268241	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000	190,000	

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Saccharin Inhibitors				
268145	N-benzoylsaccharin	15,000	45,000	16,000		
268147	N-phenylacetylsaccharin	11,000	31,000	1,300		
268146	N-diphenylacetylsaccharin	10,000	14,000	9,800		
268141	N-furoylsaccharin	22,000	39,000	20,000		
268385	N-cyanomethylsaccharin	NI[h]				

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM; human skin chymase, 0.07 μM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), human skin chymase and RMCP II were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Inhibitor concentrations were in the range 5.2 μM-1000 μM. N-Cyanomethyl saccharin was inactive at 1 mM.

[c]Inhibitor concentrations were in the range: 4.8 μM-1000 μM.

[d]Inhibitor concentrations were in the range: 3-148 μM.

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

[e]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 45 μM; 7-amino-4-chloro-3-cyclohexylmethoxyIC, 0.44 mM; Suc-Val-Pro-Phe$^{P}(OPh)_2$, 0.54 μM.

[f]Inhibitor concentrations were as follows: 3,4-dichloroisocoumarin, 540 μM; 3-benzyloxy-4-chloroIC, 1.0 μM.

[g]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

[h]No inhibition.

[i]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
268195	3,4-dichloroIC[d]	2,500	9,000
268119	3-chloroIC[d]	510	3,900
259666	4-chloro-3-(2-phenylethoxy)IC		
268440	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
268715	4-chloro-3-methyl IC	NI[e]	72%[f]
BM06288	4-chloro-3-methoxy IC	601	87
	Miscellaneous Inhibitors		
015392	isatoic anhydride		
268142	di(4-isovaleroylphenyl)sulfide		2 μM[g]
099874	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Benzoxazinones		
268398	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	216	NI
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Saccharin Inhibitors		
268145	N-benzoylsaccharin	5.2 μM[h]	2.4 μM[h]
268147	N-phenylacetylsaccharin		
268146	N-diphenylacetylsaccharin		
268141	N-furoylsaccharin	0.58 μM[h]	0.36 μM[h]
268385	N-cyanomethylsaccharin		
	Phosphonate Inhibitors		
268143	Z-Met^{P}(OPh)$_2$	NI[e]	0.8
268144	Z-Val^{P}(OPh)$_2$	2.5	90
259858	Z-Phe^{P}(OPh)$_2$	NI	6
268132	Z-Phe-Phe^{P}(OPh)$_2$	NI	NI
268241	Suc-Val-Pro-Phe^{P}(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 8-12 % Me_2SO at 25 °C. Enzyme concentrations were: PPE, 1.6 μM; HLE, 0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were in the range: 3-1000 μM.

[c]Inhibitor concentrations were in the range: 1.2-1000 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

[e]No inhibition.

[f]Inhibition was not time dependent.

[g]Inhibition was not time dependent and the IC_{50} was obtained.

[h]IC_{50} values obtained from Zimmerman, M., Morman, H., Mulvey, D., Jones, H, Frankshun, R. and Ashe, B. M. (1980) J. Biol. Chem. **255**, 9848-9851.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Isocoumarin Derivatives					
268195	3,4-dichloroisocoumarin	200	190	610		70
268397	4-chloro-3-(3-isothioureido-propoxy)isocoumarin	46,000	260,000	650,000	83,000	13,000
	Guanidinobenzoic Acid Derivatives					
268476	*p*-guanidinobenzoic acid	NI[g]				
184335	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7		NI[g]
268570	N-(*p*-guanidinobenzoyl) valine amide	4.4	4.7	1.3	2,000	
268596	O-(*p*-guanidinobenzoyl) glycolamide	100	19%[h]	130,000	5.8	NI[g]

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
BM06304	4-cyanophenyl *p*-guanidinobenzoate	150,000	91,000			
	Phosphonate Inhibitors					
268694	Z-NHCH(AmPh)PO_3Ph_2[i]	2,000		16		
	Benzamidine Derivatives	K_I (μM)	% Inhibition[j]			
BM06804	1-(amidinophenyl)-3-phenylurea	23	41			
BM06840	1-(amidinophenyl)-3-(4-chlorophenyl)urea	18	51			
BM07481	1-(amidinophenyl)-3-benzylurea	19	49			
GP8	1-(amidinophenyl)-3-(4-phenoxyphenyl)urea	1.6	71			
GP9	(4-amidinobenzyl)benzyl ether	47	31			

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Miscellaneous Inhibitors					
BM07490	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole	14,000	600			
MA-76	1-benzoyloxy-1,2,3-benzotriazole	1,100				
BM07132	N-(2-isothiureidoethyl)pthalimide	NI				

[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM and 0.02 μM; human r-*t*-PA, 0.017 μM Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase, human skin tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; *p*-guanidinobenzoic acid, 0.44 mM; ethyl *p*-guanidinobenzoate, 0.43 mM; *p*-guanidinobenzoyl valine amide,0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 mM; 4-cyanophenyl *p*-

Table III (Continued).

guanidinobenzoate, 0.46 μM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 1.22 μM; 1-benzoyloxy-1,2,3-benzotriazole, 22 μM.

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 μM; ethyl *p*-guanidinobenzoate, 0.42 mM; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.42 mM; 4-cyanophenyl *p*-guanidinobenzoate, 0.22 μM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 11.6 μM.

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 μM; ethyl *p*-guanidinobenzoate, 0.45 mM ; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 μM.

[e]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.35 μM; *p*-guanidinobenzoyl valine amide, 3.5 μM; O-(*p*-guanidinobenzoyl)glycolamide, 1.7 mM.

[f]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 4.3 μM; ethyl *p*-guanidinobenzoate, 0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.43 mM.

[g]No inhibition.

[h]Inhibition was not time dependent and the % inhibition was measured at 0.42 mM.

[i] AmPh = 4-amidinophenyl

Table III (Continued).

jInhbitior concentrations were 226 μM.

Animal Testing Priorities

The various compounds which we have submitted have been prioritized for animal testing and the following table lists the priority which we have assigned to each compound and the reasons for that priority.

High-1 WR268195 3,4-dichloroisocoumarin
This isocoumarin is a general serine protease inhibitor and effectively inhibits most of the enzymes tested.

High-2 GP8 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea
This benzamidine should be very stable in solution and is an excellant competitive inhibitor of trypsin-like enzymes.

High-3 WR268241 Suc-Val-Pro-Phe^{P}(OPh)$_2$
This peptide phosphonate is a reactive and specific inhibitor for chymotrypsin-like enzymes including chymases.

High-4 WR268397 4-chloro-3-(3-isothioureidopropoxy)isocoumarin
This isocoumarin is a very reactive inhibitor for the rat skin tryptase.

High-5 WR268398 2-ethoxy-4H-3,1-benzoxazin-4-one
This benzoxazinone inhibitor is an effective inhibitor for elastase.

High-6 WR268141 N-furoylsaccharin
This saccharin is a general protease inhibitor and inhibits elastases and chymotrypsin-like enzyme quite effectively and is probably a moderate inhibitor for trypsin-like enzymes.

High-7 BM06304 4-cyanophenyl *p*-guanidinobenzoate
This guanidinium substituted derivative is an effective inhibitor for trypsin-like enzymes

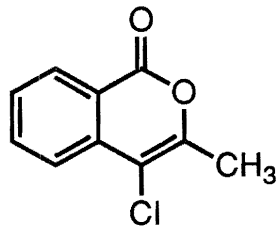
High-8 WR268440 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin
This isocoumarin is an effective elastase inhibitor.

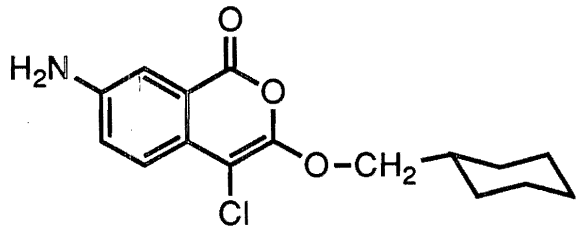
High-9 WR268142 di(4-isovaleroylphenyl)sulfide
This aromatic derivative is an effective elastase inhibitor.

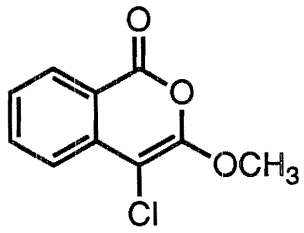
High-10 WR268387 4-chloro-3-benzyloxyisocoumarin
This isocoumarin is an effective inhibitor for chymotrypsin-like enzymes.

SYNTHESIS

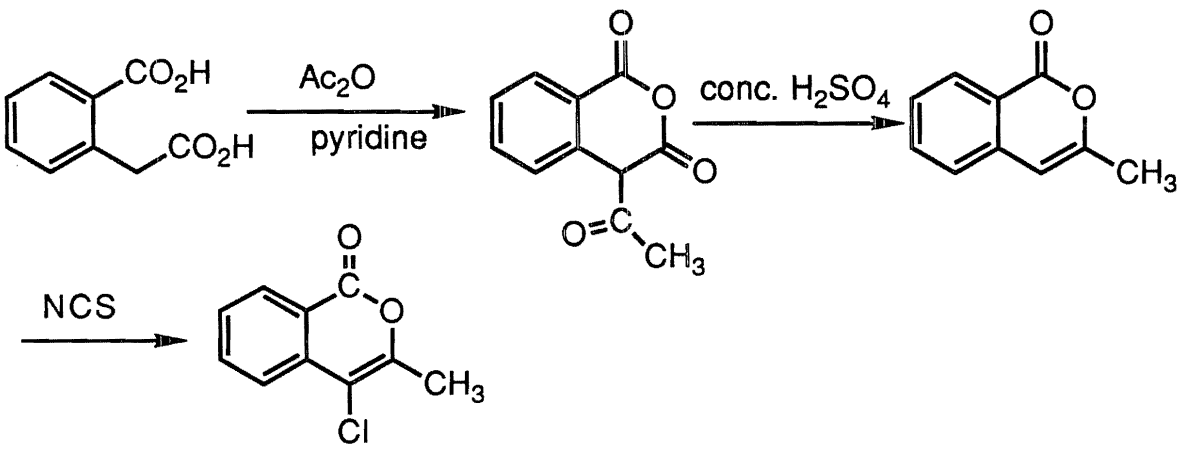
Isocoumarins. Three new isocoumarins have been synthesized this year and submitted for testing as antivesicants. The compounds are 4-chloro-3-methylisocoumarin (WR268715), and 4-chloro-3-methoxyisocoumarin (GP2), and 7-amino-4-chloro-3-cyclohexylmethoxyisocoumarin (WR268693).





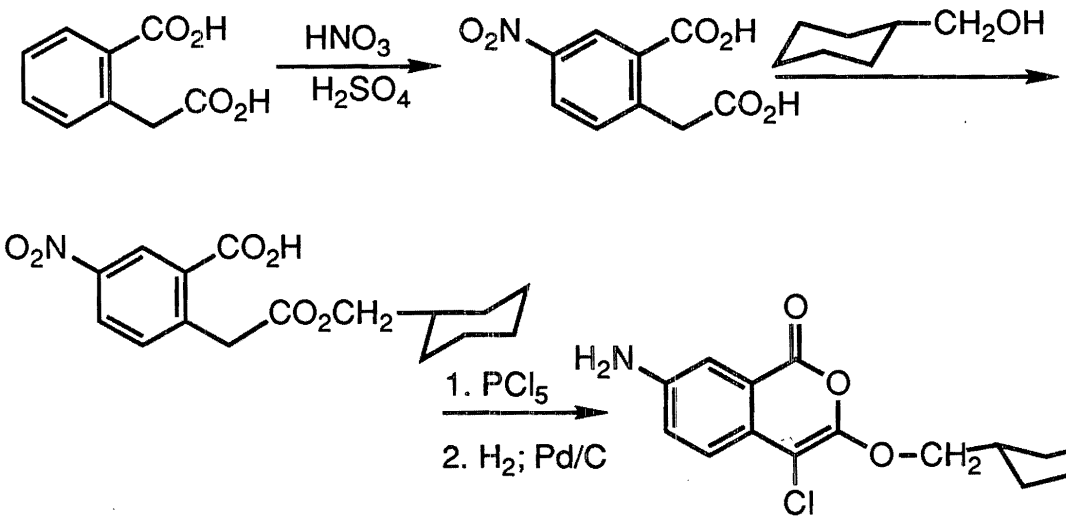


4-Chloro-3-methylisocoumarin was prepared by reaction of homophthalic acid with acetic anhydride in pyridine to obtain 4-acetyl homophthalic anhydride which was then treated with conc. H_2SO_4 to yield 3-methylisocoumarin. The latter compound gave the final product by reaction with N-chlorosuccinimide.

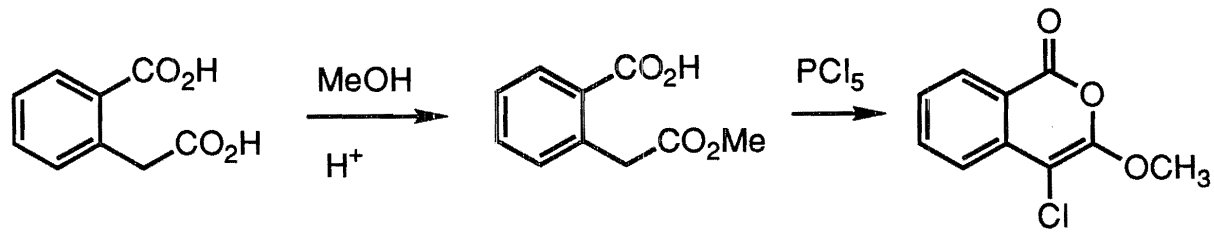


7-Amino-4-chloro-3-cyclohexylmethoxyisocoumarin was obtained by a multistep procedure involving nitration of homophthalic acid, esterification of the resulting nitrohomophthalic acid with cyclohexanemethanol to the monoester and cyclization of the latter to the 3-alkoxy-4-chloro-7-nitroisocoumarin using phosphorus pentachloride. The last step, reduction of the nitro group to the amino group can only be done on a small scale (about 1 g),

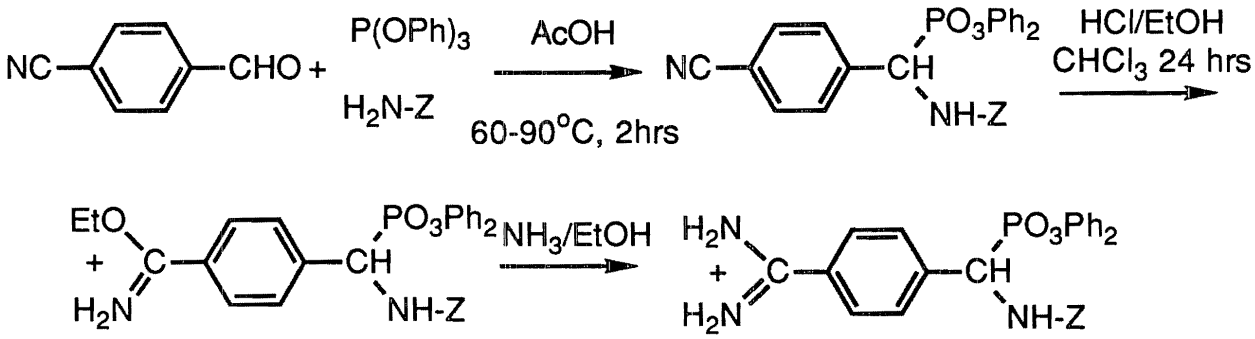
probably due to reaction of the amino group with the isocoumarin ring.



4-Chloro-3-methoxyisocoumarin was prepared by the following route.

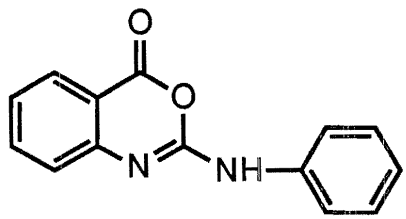


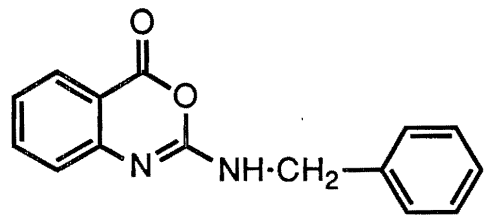
Phosphonates. Diphenyl N-benzyloxycarbonylamino(4-amidinophenyl)methane phosphonate was obtained by α-amidoalkylation of triphenyl phosphite using 4-cyanobenzaldehyde and benzyl carbamate to give diphenyl N-benzyloxycarbonylamino(4-cyanophenyl)methane phosphonate. The cyano group was then converted to an imino ester by reaction with HCl in ethanol and finally to an amidino moiety by reaction with ethanolic ammonia.



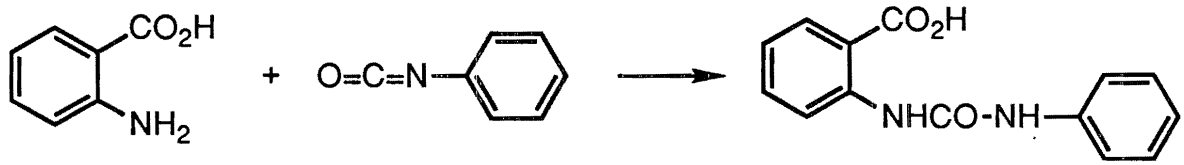
Benzoxazinones. Two new compounds have been synthesized this year and submitted for testing as antivesicants. The compounds are 2-phenylamino-4H-3,1-benzoxazin-4-one and 2-

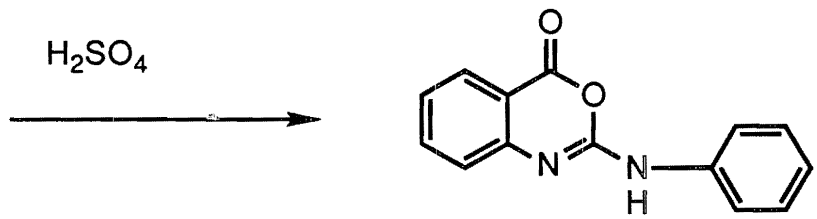
Benzylamino-4H-3,1-benzoxazin-4-one, The structures are shown below.



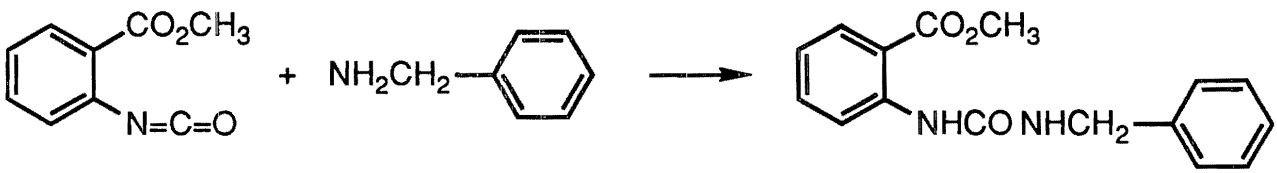


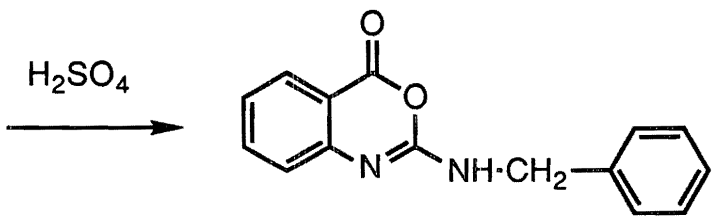
2-Phenylamino benzoxazin-4-one was prepared by the cyclodehydration of 2-(3-phenylureido)benzoic acid. The latter was prepared by the reaction of phenyl isocyanate with anthranilic acid in THF.



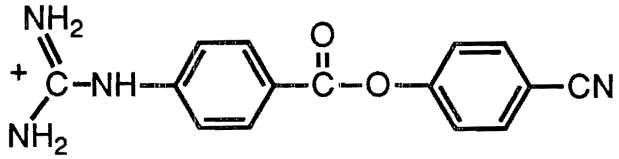


2-Benzylamino-4H-3,1-benzoxazin-4-one was also prepared by a cyclodehydration reaction using H_2SO_4 as the dehydrating agent. The starting compound for this reaction was methyl 2-(3-benzylureido)benzoate. This in turn was prepared by the reaction of 2-carbomethoxy phenylisocyanate with benzyl amine in THF at room temperature.

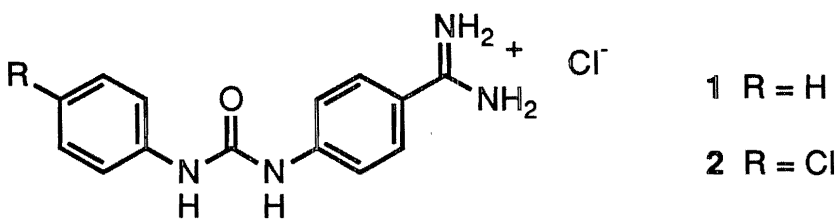




***p*-Guanidinobenzoic Acid Derivatives.** 4-Cyanophenyl *p*-guanidinobenzoate was prepared from *p*-guanidinobenzoic acid and 4-cyanophenol using DCC as the coupling agent.



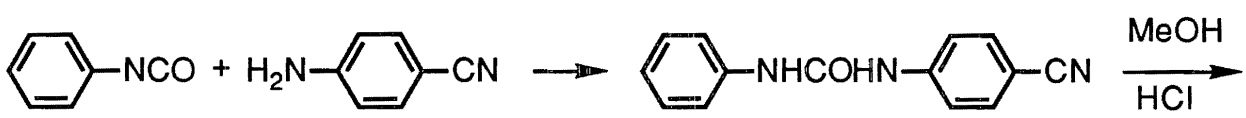
Benzamidines. The synthesis of *p*-guanidino benzoic acid derivatives was discontinued due to numerous problems in the synthetic/purification procedures when the substituents on the carboxyl group were more complex aromatic structures than a single phenyl group. We are presently concentrating on derivatives of benzamidine as inhibitors for trypsin-like enzymes. Although the synthesis and purification procedures are still difficult, better results are being obtained with more complex aromatic structures containing benzamidine-like moieties. Thus far five benzamidines have been prepared as inhibitors for trypsin-like enzymes. Two representative compounds are 1-(4-amidinophenyl)-3-phenylurea hydrochloride (**1**, BM06804) and 1-(4-amidinophenyl)-3-(4-chlorophenyl)urea hydrochloride (**2**, BM06840).

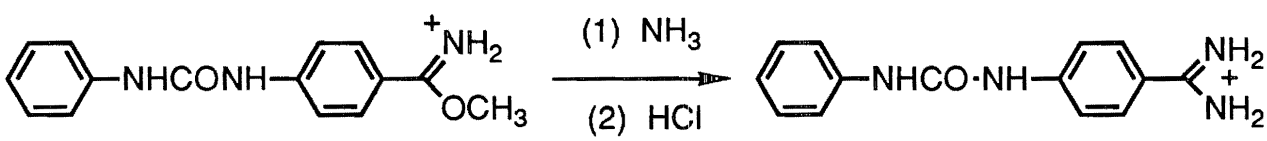


1 R = H

2 R = Cl

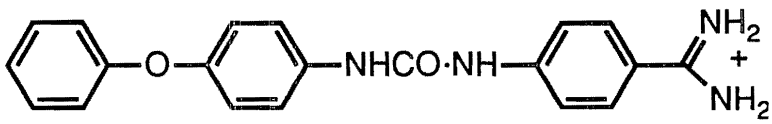
Numerous attempts to make 1-(4-amidinophenyl)-3-phenylurea (**1**) and its 4-chloro analog (**2**) by condensing 4-aminobenzamidine with phenylisocyanate and 4-chlorophenylisocyanate failed. These compounds were finally made by a different route. 1-(4-Amidinophenyl)-3-phenylurea (**1**) was finally prepared starting from 4-aminobenzonitrile. Phenylisocyanate and 4-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by treatment with dry methanol in the presence of dry HCl. The final amidino compound was obtained by refluxing the imidate ester in isopropanol saturated with dry ammonia. The product was isolated as its hydrochloride salt and was submitted this year.





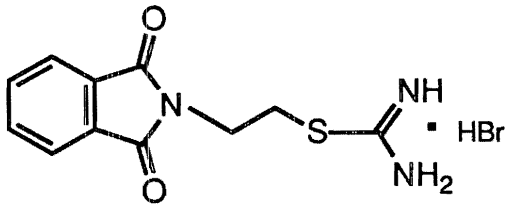
1-(4-Amidinophenyl)-3-(4-chlorophenyl)urea (**2**) was prepared using the same reaction scheme except that phenylisocyanate was replaced by 4-chlorophenylisocyanate.

1-(4-Amidinophenyl)-3-benzylurea (BM07481) and 1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea (GP8, below) were made using essentially the same set of reactions. Any modifications which were required in going from one product to the other are clearly noted in the Experimental Section.

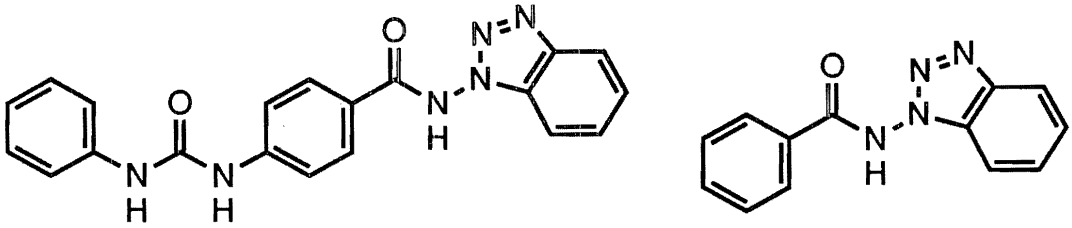


Micellaneous Compounds. Several new compounds with a variety of structures were synthesized this year and submitted for testing as antivesicants.

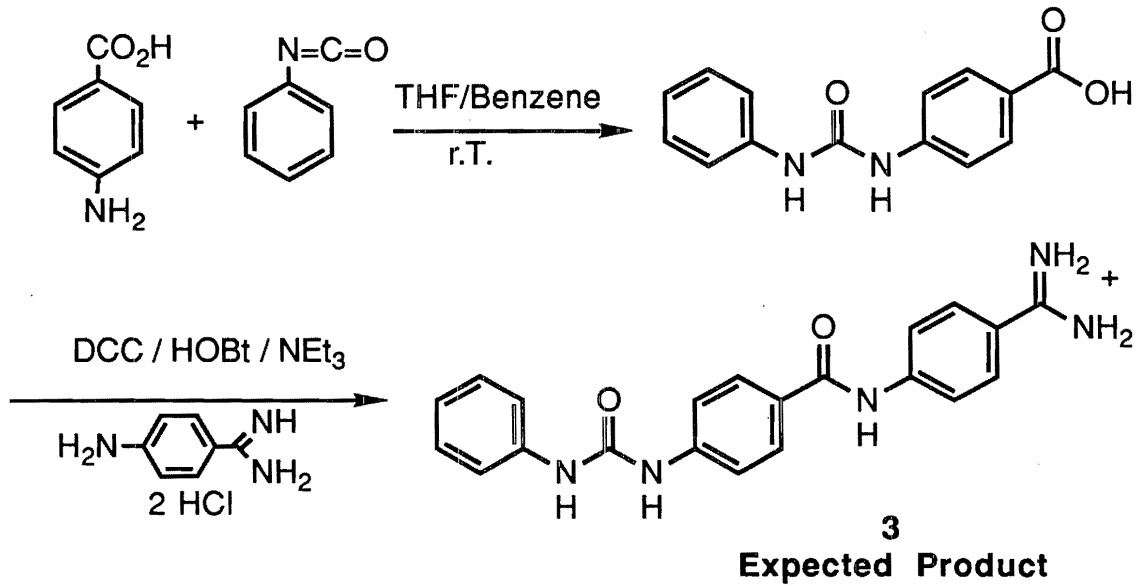
One structure was N-(2-isothiureido)ethyl phtalimide hydrobromide which was prepared by alkylation of thiourea with N-(2-bromoethyl)phtalimide in THF. This compound was modeled after the isothiureido isocoumarins prepared in our laboratories which have shown significant inhibitory potency towards trypsin and trypsin-like enzymes.



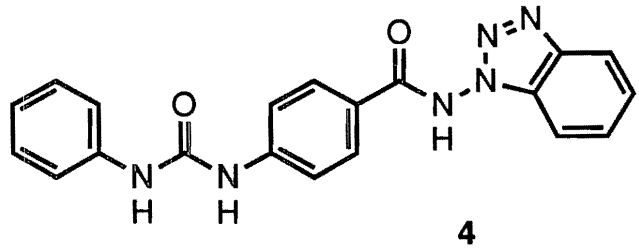
Two N-hydroxybenzotriazole derivatives were synthesized. They are 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole (BM07490) and 1-benzoyloxy-1,2,3-benzotriazole (MA-76). Their structures are shown below.



The first derivative was reported as N-(4-amidinophenyl)-4-(phenylcarbamoylamino)benzamide in one of the quarterly reports. The synthesis involved a DCC coupling of 4-(phenylcarbamoylamino)benzoic acid with 4-aminobenzamidine using 1-hydroxybenzotriazole in DMF. The acid was prepared from 4-aminobenzoic acid and phenyl isocyanate as depicted below.



3
Expected Product



4
Actual Product

At that time the compound was reported as a mixture of salts because the elemental analysis could not be matched with the expected structure (**3**). The fact that it was a good trypsin and human lung tryptase inhibitor further suggested that it must contain the amidino moiety in its structure. On the other hand, all the analytical data could be explained by a compound with the structure corresponding to an acylated benzotriazole, namely 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole (**4**). The compound is therefore reported as such in the Experimental Section. A spectrophotometric study of the hydrolysis of this compound in the buffer used to assay inhibition of trypsin shows at least two isosbestic points over a 5 h period. However, no significant hydrolysis was observed during the short time periods used in the inhibition assays and therefore the inhibition constants reported are accurate values. Kinetic studies are being performed to elucidate a mechanism for the inhibition of trypsin by this compound. The benzoyl derivative was made in order to compare its inhibition of trypsin with that of the more complex derivative discussed above. It was also made in order to ascertain if an acyl derivative of 1-hydroxy-1,2,3-benzotriazole could indeed inhibit trypsin. We found this to be the case, and 1,2,3-

benzotriazole-1-benzoate had a $k_{obs}/[I]$ value of 1,100 $M^{-1}s^{-1}$ towards trypsin. It was made by acylation of 1-hydroxy-1,2,3-benzotriazole with benzoyl chloride in THF/pyridine. This type of inhibitors are a new and interesting class of trypsin inhibitors and mechanistic studies will help us elucidate their mode of action.

EXPERIMENTAL SECTION

Cyclohexanemethyl 4-nitro-2-carboxyphenylacetate. A solution containing 10 g (44 mmol) of 5-nitro homophtalic acid [prepared according to H. E. Ungnade, D.V. Nightingale and H. E. French (1945) J. Org. Chem. **10**, 533], cyclohexanemethanol (14 mL, 111 mmol) and 6 drops of conc. H_2SO_4 in 150 mL of benzene was heated to reflux temperature with azeotropic removal of water for 4.5 h. The reaction mixture was cooled to room temperature and then extracted with a saturated solution of $NaHCO_3$ (5 x 25 mL) and water (2 x 25 mL). Excess concentrated HCl was added to the aqueous extract and the white solid which precipitated was collected by filtration and dried to yield 8.58 g (61%) of product that was pure enough to be used in the next step; m.p. 141-2 °C.

4-Chloro-3-cyclohexylmethoxy-7-nitroisocoumarin. A solution of cyclohexanemethyl 4-nitro-2-carboxyphenylacetate (8.58 g, 27 mmol) and PCl_5 (13.9 g, 67 mmol) in 100 mL of benzene was heated to reflux temperature for 4 h and then the solvent was removed *in vacuo*. The residue was recrystallized from 95% EtOH to yield the product as bright yellow needles (5.13 g, 56%); m.p. 102-104 °C.

7-Amino-4-chloro-3-cyclohexylmethoxyisocoumarin. Palladium on charcoal (150 mg, 5% catalyst) was added to a solution of 4-chloro-3-cyclohexylmethoxy-7-nitroisocoumarin (1.24 g, 3.7 mmol) in 200 mL of EtOAc and the resulting mixture was stirred under a hydrogen atmosphere for 6 h at atmospheric pressure. The catalyst was then removed by filtration through a bed of Celite and the filtrate was concentrated to dryness *in vacuo*. The residue was

recrystallized from ethanol/water to yield the product as bright yellow needles (801 mg, 71%); m.p. darkens >180 °C. ^{1}H NMR (d_6-$MeSO_4$) δ: 7.4 (d, 1H), 7.25 (d, 1H), 7.14 (d of d, 1H), 5.76 (br. s, 2H), 4.05 (d, 2H), 1.76-1.58 (m, 6H), 1.30-0.98 (m, 5H). Anal Calcd. for $C_{16}H_{18}ClNO_3$: C, 62.44; H, 5.89; N, 4.55; Cl, 11.52. Found: C, 62.50; H, 5.93; N, 4.51; Cl, 11.42.

Diphenyl N-benzyloxycarbonylamino(4-cyanophenyl)-methanephosphonate. Obtained from 9.75 g 4-cyano-benzaldehyde, 7.65 g benzyl carbamate and 13.5 ml of triphenyl phosphite in 20 ml of glacial acetic acid, according to procedure described earlier [Oleksyszyn, J., Subotkowska, L., Mastalerz, P. (1979) Synthesis, 985]. Yield 70%; mp. 135-138 °C; Anal. Calcd. for $C_{28}H_{23}O_5N_2P$. $^1/_2$ H_2O: C, 66.27; H, 4.73; N, 5.52. Found; C, 66.03; H, 4.51; N, 5.49.

Diphenyl N-benzyloxycarbonylamino(4-amidinophenyl)-methanephosphonate hydrochloride. A solution of 7 g diphenyl Z-benzyloxycarbonylamino(4-cyanophenyl)-methanephosphonate in 150 mL of dry chloroform and 15 mL of absolute ethanol was saturated with dry HCl at 0 °C. The reaction mixture was kept in the refrigerator until TLC showed no presence of starting material (about 24 h). An excess of pentane was added and the precipitate was removed by filtration and dried under high vacuum. The solid was dissolved in 200 ml of dry methanol and gaseous dry ammonia was bubbled through the solution (one equivalent is required) for approximately 20 min. Methanol and excess ammonia were removed as fast as possible in the rotary evaporator. A portion of fresh methanol (100 mL) was added and the solution was heated at 50 °C by about 8 h, until TLC shows no presence of imino ether. The solvent was evaporated and the

resulting oil was dissolved in chloroform. Addition of ether caused separation of an oil which solidified after a while. The solid that resulted was again dissolved in chloroform, the solution was filtered and the solid product was precipitated using ether. In several experiments the yields were 70-80%; mp. 154-158 °C (decomp); ^{31}P NMR 14,87 ppm. Anal. Calcd. for $C_{28}H_{27}O_5N_3ClP \cdot 0.3NH_4Cl \cdot H_2O$; C, 57.41; H, 5.16; N, 7.52; Cl, 7.31. Found; C, 57.75; H, 5.00; N, 8.86; Cl, 7.43.

Diphenyl amino(4-amidinophenyl)methanephosphonate dihydrochloride. A sample of 1.8 g of diphenyl N-benzyloxy-carbonylamino(4-amidinophenyl)methanephosphonate hydrochloride was dissolved in 150 mL of 2N HCl/methanol solution and after addition of 5% Pd/C catalyst the resulting mixture was stirred under an atmosphere of hydrogen until the theoretical amount of hydrogen was consumed. The catalyst was removed by filtration and after evaporation of methanol a residue was crystallized from ethanol-ether. In several experiments the yields were 60-80%; mp. 213-215 °C; Anal. Calcd. for $C_{20}H_{22}N_3ClP \cdot {}^1/_2H_2O$: C, 51.85; H, 4.97; N, 9.08; Cl, 15.34. Found: C, 51.73; H, 5.02; N, 9.10; Cl, 15.36.

4-Chloro-3-methylisocoumarin. To a stirred solution of 10 g (0.06 mol) of 3-methylisocoumarin [Tirodkar, R.B. and Usgaonkar, R. N. (1969) J. Ind. Chem. Soc. **46**, 935) in 25 mL of DMF was added 11.16 g (0.08 moles) of N-chlorosuccinimide and the reaction mixture stirred at room temperature overnight. The reaction mixture was then diluted with ether and the ether layer was washed successively with 10 % HCl, 5 % $NaHCO_3$, water and finally dried over anhydrous $MgSO_4$. Ether was evaporated and the solid obtained was

recrystallized from hexane to give 3.5 g (29 %) of the title compound as yellowish needles; m.p. 91-93 °C; H^1 NMR ($CDCl_3$) δ: 2.48 (s,3H), 7.55 (m,1H), 7.80 (m,2H), 8.31 (d,1H). Anal. Calcd. for $C_{10}H_7ClO_2$ 0.2 H_2O : C, 60.54; H, 3.53; Cl, 17.91. Found : C, 60.68; H, 3.66; Cl, 17.98.

2-Phenylamino-4H-3,1-benzoxazin-4-one. Phenyl isocyanate (3.57 g, 0.03 mol) was dissolved in 10 mL THF and to this stirred solution was added anthranilic acid (4.1 g, 0.03 mol) and the reaction stirred overnight at room temperature. The solvent was then removed under reduced pressure to get 2-(3-phenylureido) benzoic acid as a white solid.

The product from the above reaction (3.84 g) was dissolved in 10 mL conc. H_2SO_4 and kept at room temperature for 1 h. The reaction was then poured over ice, neutralized with saturated $NaHCO_3$ solution, the precipitated white solid filtered and dried in air and recrystallized from benzene to get the title compound as white crystals; m.p. 191-192 °C. ^1H NMR (DMSO-d_6) δ: 7.1-8.0 (aromatic). Anal. Calcd.for $C_{14}H_{10}N_2O_2$ 0.27 H_2O : C, 69.13; H, 4.12; N, 11.52. Found: C, 69.10; H, 4.32; N, 10.71.

2-Benzylamino-4H-3,1-benzoxazin-4-one. To a stirred solution of benzylamine (634 mg, 0.0059 mol) in dry THF (40 mL) was added 2-carbomethoxy phenylisocyanate (1 g, 0.0056 mol) and the reaction stirred at room temperature overnight. The solvent was removed under reduced pressure to obtain methyl 2-(3-benzylureido) benzoate as a white solid.

The product (1.13 g) from the reaction above was dissolved in 2 mL conc. H_2SO_4 and left at room temperature for 1 h. The reaction

was worked up as in the previous experiment and the product recrystallized from ethyl acetate-benzene to get the title compound as a white solid; m.p. 177-178 °C. ^{1}H NMR (DMSO-d_6) δ: 4.3 (d, 2H, -CH_2-), 6.9-8.4 (aromatic), 10.2 (s, 1H, NH). Anal. Calcd. for $C_{15}H_{12}N_2O_2$ x 1.1 H_2O: C, 66.17; H, 5.22; N, 10.29. Found: C, 66.20; H, 5.24; N, 10.15.

4-Cyanophenyl *p*-Guanidino Benzoate. Dicyclohexyl carbodiimide (2.33 g, 11.3 mmol) was added to a solution of *p*-guanidinobenzoic acid hydrochloride (2.43 g, 11.3 mmol) in 50 mL pyridine. 4-Cyanophenol (1.34 g, 11.3 mmol) was added to this solution and the resulting mixture was stirred at room temperature for 14 h with protection from moisture. Dicyclohexylurea was removed from the reaction mixture by filtration and the filtrate was concentrated *in vacuo* to a foam which was triturated with 30 mL EtOAc. The beige solid that resulted was collected by filtration and washed throughly with EtOAc. The crude product (2.5 g) is taken up in approx. 3 L of water and the solid remaining is removed by filtration through a bed of Celite. The filtrate was lyophilized to obtain 1.75 g (70 %) of pure product; m.p. 160-62 °C. ^{1}H NMR (d_6-Me_2SO) δ: 10.58 (s, 1H), 8.18 (d, 2H), 7.99 (d, 2H), 7.88 (br.s., 3H), 7.52 (d, 2H), 7.42 (d, 2H). Anal. Calcd. for $C_{15}H_{13}ClN_4O_2$ x 1.98 H_2O: C, 51.13; H, 4.85; N, 15.90. Found: C, 51.13; H, 4.70; N, 15.79.

1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole. Triethylamine (0.19 mL, 1.4 mmol) was added to a solution of 4-aminobenzamidine dihydrochloride (284 mg, 1.4 mmol) in 6 mL DMF. 1,3-Dicyclohexylcarbodiimide (311 mg, 1.5 mmol) was then added,

followed by 1-hydroxy-1,2,3-benzotriazole (185 mg, 1.4 mmol). This mixture was cooled to 10 °C and 4-phenylaminocarbamoyl benzoic acid was then added (350 mg, 1.4 mmol). The reaction mixture was stirred at 5-10 °C for 14 h and dicyclohexylurea was removed by filtration. It was washed with fresh DMF, and the filtrate was concentrated to dryness and coevaporated with acetone to obtain an amorphous white solid that was washed with $CHCl_3$:EtOH 4:1 to obtain 225 mg (40 %) of pure product as a white solid; mp 217-8 °C (dec). The product can be recrystallized from $CHCl_3$/DMF/hexane. ^1H NMR (DMSO-d_6) δ: 9.45 (br s, 1H); 8.96 (br s, 1H); 8.22 (d, 2H); 8.18 (d, 1H); 7.85 (d, 1H); 7.78 (d, 2H); 7.67 (t, 1H); 7.54 (t, 1H); 7.49 (d, 2H); 7.32 (t, 2H); 7.02 (t, 1H). Anal Calcd. for $C_{20}H_{15}N_5O_3 \cdot 0.2\ H_2O$: C, 63.71; H, 4.12; N, 18.58. Found: C, 63.71; H, 4.36; N, 18.80.

N-(2-Isothiureido)ethyl phtalimide hydrobromide. N-(2-Bromoethyl)phtalimide (4.6 g, 18 mmol) was dissolved in 50 mL THF and then thiourea (1.52 g, 20 mmol) was added in one portion. The resulting mixture was heated to reflux temperature for 2 days and then allowed to cool to 5-10 °C in an ice bath. The white solid in suspension was filtered and washed with THF and hexane to yield 3.21 g (54%) of pure product; mp 245-6 °C (dec). ^1H NMR (DMSO-d_6) δ: 9.04 (br s, 4H); 7.86-7.91 (m, 4H); 3.87 (t, 2H); 3.49 (t, 2H). Anal. Calcd. for $C_{11}H_{12}BrN_3O_2S$: C, 40.01; H, 3.66; N, 12.73; Br, 24.20. Found: C, 40.10; H, 3.71; N, 12.72; Br, 24.14.

1-Benzoyloxy-1,2,3-benzotriazole. A solution of benzoyl chloride (6.2 mL, 53 mmol) in 30 mL of THF was added dropwise to a solution of 1-hydroxy-1,2,3-benzotriazole (6.5 g, 48 mmol) and pyridine (4.3 mL, 53 mmol) in 65 mL THF. The resulting mixture was

stirred at room temperature for 24 h under protection from moisture, and the solvent was then removed under vacuum. The white solid obtained was purified by low temperature recrystallization from $CHCl_3$/hexane and cooling at 0-5 °C for 2 days. The pure product was obtained as a white solid (5.15 g, 47 %); mp 78-9 °C. A second crop can be obtained from the mother liquor by concentration to 2/3 of the volume, addition of hexane, and further cooling at 0-5 °C for 2 days. ^1H NMR (DMSO-d_6) δ: 7.98-7.92 (m, 3H); 7.72 (d, 1H); 7.64-7.38 (m, 5H). Anal. Calcd. for $C_{13}H_9N_3O_2$: C, 65.26; H, 3.79; N, 17.56. Found: C, 65.36; H, 3.81; N, 17.61.

1-(4-Amidinophenyl)-3-phenylurea. To a stirred solution of 4-aminobenzonitrile (4.72 g, 0.04 mol) in dry benzene (200 mL) was added phenylisocyanate (9.52 g, 0.08 mol) and the resulting solution refluxed for 5 h and further stirred at room temperature overnight. The precipitated solid was filtered and recrystallized from methanol to get the urea as a white crystalline solid (8.3 g).

Dry HCl gas was passed through a solution of the urea (4.74 g, 0.02 mol) in dry dimethoxyethane (100 mL) for 1 h. The reaction mixture was then stirred at room temperature for 24 h. The precipitated imidate ester was filtered and stored over KOH in a vacuum desiccator (3.5 g) for 24 h.

The imidate ester (2 g, 0.006 mol) was dissolved in isopropanol saturated with ammonia (100 mL) and the reaction mixture was refluxed for 6 h and further stirred at room temperature overnight. The solvent was then evaporated under reduced pressure, a white solid that was obtained, and this was recrystallized from 2N HCl to get the title compound as its HCl salt (1.5 g); m. p. 240 °C

(decomp.). ^{1}H NMR (DMSO-d_6) δ: 7.00 (t, 1H), 7.30 (t, 2H), 7.45 (d, 2H), 7.65 (d, 2H), 7.80 (d, 2H), 8.80 (s, 2H), 9.15 (s, 2H), 9.40 (bs, 1H), 9.80 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}ClN_4O \cdot H_2O$: C, 54.53; H, 5.56; Cl, 11.35; N, 18.18. Found: C, 54.53; H, 5.46; Cl, 11.42; N, 18.13.

1-(4-Amidinophenyl)-3-(4-chlorophenyl)urea hydrochloride. To a stirred solution of 4-aminobenzonitrile (4.72 g, 0.04 mol) in dry THF (50 mL) was added 4-chlorophenylisocyanate (6.12 g, 0.04 mol) in one portion and the reaction stirred magnetically overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (9.2 g, 85 %).

Dry HCl was passed through a cooled solution of the cyano urea (5.43 g, 0.02 mol) and dry methanol (3.2 g, 0.10 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction further stirred at room temperature for 36 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained as a yellow solid was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (6 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (4.8 g, 84 %); mp 275 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 7.32 (d, 2H); 7.50 (d, 2H); 7.66 (d, 2H); 7.80 (d, 2H); 8.85 (s, 2H); 9.18 (s, 2H); 9.70 (s, 1H); 9.98 (s, 1H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O \cdot 0.69\ H_2O$: C, 49.80; H, 4.58; Cl, 21.01; N, 16.59. Found: C, 50.21; H, 4.62; Cl, 20.73; N, 16.19.

1-(4-Amidinophenyl)-3-benzylurea hydrochloride. To a stirred solution of 4-aminobenzonitrile (3.9 g, 0.03 mol) in dry benzene (100 mL) was added benzylisocyanate (4.5 g, 0.03 mol) in one portion and the reaction heated under reflux for 6 h and stirred magnetically at room temperature overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (6.1 g, 72 %).

Dry HCl was passed through a cooled solution of the cyano urea (3 g, 0.012 mol) and dry methanol (1.92 g, 0.060 mol) in dry dimethoxyethane (150 mL) for 2 h and the reaction further stirred at room temperature for 24 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained as a yellow solid was stored in a vacuum desiccator over KOH for 24 h (3.2 g).

The imidate ester (3.2 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained recrystallized from 2N HCl to get white crystals of the title compound (3 g, 84 %); mp 292 °C (dec.). ^{1}H NMR (DMSO-d_6) δ: 4.31 (d, 2H); 7.17 (t, 1H); 7.20-7.38 (m, 5H); 8.82 (s, 2H); 9.12 (s, 2H); 9.66 (s, 1H). Anal. Calcd. for $C_{15}H_{17}Cl_2N_4O$: C, 59.11; H, 5.62; Cl, 11.63; N, 18.38. Found: C, 59.23; H, 5.64; Cl, 11.63; N, 18.28.

1-(4-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride. To a stirred solution of 4-aminobenzonitrile (2.95 g, 0.025 mol) in dry benzene (50 mL) was added 4-phenoxyphenylisocyanate (5.25 g, 0.025 mol) in one portion and the reaction heated under reflux for 10 h and stirred at room

temperature overnight. The separated white solid was filtered out and recrystallized from methanol to get the cyano urea as a white crystalline solid (6.5 g, 79 %).

Dry HCl was passed through a cooled solution of the cyano urea (5 g, 0.015 mol) and dry methanol (2.43 g, 0.075 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction stored in the refrigerator for 48 h. Solvent was then evaporated under reduced pressure and the solid obtained stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (5 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 7 h and further stirred at room temperature for 24 h. Solvent was evaporated and the solid obtained recrystallized from 2N HCl to get white crystals of the title compound (4.6 g, 95 %); mp 202-205 °C. ^{1}H NMR (DMSO-d_6) δ: 6.90-7.01 (m, 4H); 7.09 (t, 1H); 7.35 (m, 2H); 7.47 (m, 2H); 7.65(d, 2H); 7.80 (d, 2H); 8.80 (s, 2H); 9.15 (s, 2H); 9.39 (s, 1H); 9.75 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.7\ H_2O$: C, 60.74; H, 5.16; Cl, 8.98; N, 14.17. Found: C, 60.36; H, 5.05; Cl, 9.00; N, 14.08.

4-Chloro-3-methoxyisocoumarin. This compound was prepared according to the procedure of Tirodkar, R. B., and Usgaonkar, R.N. (1969) Indian J. Chem. **7**, 1114; m.p. 91-93 °C. Anal. Calcd.for $C_{10}H_7Cl_1O_3 \cdot 0.1\ H_2O$: C, 56.65; H, 3.31; Cl, 16.76; Found: C, 56.45; H, 3.46; Cl, 16.61.

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PROTEASE INHIBITORS AS ANTIVESICANTS

JAMES C. POWERS

School of Chemistry
Georgia Institute of Technology
Atlanta, GA 30332

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19. ABSTRACT

Sulfur mustard is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis. The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister. A total of 45 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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ABSTRACT

Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. The mustard is thought to alkylate DNA which eventually results in the release of potent proteolytic (protein degrading) enzymes, particularly serine proteases. These serine proteases are capable of destroying a number of connective tissue proteins and several of the enzymes seem to be able to specifically cleave proteins at the junction of the dermis and epidermis (outer non-vascular layer of skin). The destruction of these basement membrane and connective tissue proteins results in an epidermal-dermal separation, fluid accumulation, and formation of a blister.

A total of 45 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 11 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. The inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

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BACKGROUND

Sulfur Mustard. Bis-(2-chloroethyl)sulfide (HD, sulfur mustard) is a potent vesicant which was used in the Iran-Iraq conflict. Sulfur mustard penetrates the skin rapidly and after a latent period of several hours under temperate climatic conditions, causes extensive blistering of the skin. Antimustard ointments which attempt to inactivate the free mustard are ineffective since the mustard quickly reacts with components in the skin and other tissues. Therefore, an effective therapy for sulfur mustard exposure must be based on reversing the physiological processes that result upon contact with this potent vesicant (Cullumbine, 1947).

Bis-(2-chloroethyl)sulfide is a potent alkylating agent which can react with the heterocyclic bases in nucleic acids (Ludlum et al., 1984) and with a wide variety of the side chain functional groups (thiols, thioethers, carboxyl groups, amino groups, imidazole rings, etc.) found in proteins. The majority of the sulfur mustard is secreted in the form of various metabolites such as thiodiglycol, but some is carried by the circulation to other organs, and a significant portion is stored in skin reservoirs (Klain and Bonner, 1987). While the most significant mustard induced injury occurs in the skin (vesication and inflammation), significant numbers of mustard casualties have ocular injuries and cornea impairment for 2-4 months. In cases of severe exposure, there is serious lung and bone marrow damage which results in death.

Mechanism of Sulfur Mustard Induced Blistering. The molecular mechanisms by which sulfur mustard causes toxicity are unknown but mustard is a powerful alkylating agent of DNA and RNA. Papirmeister has suggested that the alkylated purine bases in DNA are unstable and undergo both spontaneous and enzymatic depurination (Papirmeister et al., 1985). This results in DNA strand breaks, and activation of nucleases and other DNA repair mechanisms. As a result, poly(ADP-ribose)polymerase is activated, NAD^+ is depleted, glycolysis is inhibited, and the hexose monophosphate shunt is stimulated (Meier et al., 1987). This causes the release of potent proteolytic enzymes which produces the observed pathology of basal cell necrosis and vesication.

Evidence for the Papirmeister hypothesis includes the isolation and structural characterization of several DNA alkylation products upon treatment of DNA with sulfur mustard (Benschop et al., 1989) and the demonstration of single strand breaks in the DNA after exposure of keratinocyte cultures to low levels of sulfur mustard (Bernstein et al., 1989). In addition, other agents which result in DNA damage such as UV light and radiation have been shown to stimulate the synthesis or release of proteases in fibroblast cultures (Miskin and Reich, 1980).

Proteases are normally controlled by natural plasma protein protease inhibitors such as α_1-protease inhibitor, α_1-

antichymotrypsin, and α_2-macroglobulin. If this antiprotease screen is destroyed, tissue destruction results. Several of the plasma serpins (serine protease inhibitors) including α_1-protease inhibitor and α_1-antichymotrypsin have essential methionine residues and are susceptible to inactivation by oxidizing agents or alkylating agents. A single dose of sulfur mustard in the mouse brain has recently been shown to cause a burst of oxidants (Elsayed et al., 1989). The serpin screen could then be removed directly by sulfur mustard alkylation or indirectly by oxidation as a result of this oxidative burst. Thus, sulfur mustard exposure probably results both in the release of powerful proteolytic enzymes and in the partial destruction of the protease inhibitor screen which would normally protect the organism from proteolysis.

Other Blistering Disease States Involve Proteases. Blistering disease states which have been described include dermatitis herpetiformis (DH), bullous pemphigoid (BP), chronic bullous disease of childhood, and pemphigus vulgaris. These diseases are characterized by destruction of various connective tissue components of the epidermis or dermis followed by tissue separation and the formation of fluid-filled blisters. Blister fluids from patients with all of these diseases have been shown to contain proteases including elastase and collagenase (Oikarinen et al., 1983). Human polymorphonuclear leukocyte elastase is the major enzyme in DH fluid, while BP fluid predominantly contains the metalloprotease collagenase. A trypsin-like enzyme and a thiol protease have also been implicated in blister formation respectively in recessive dystrophic epidermolysis bullosa and epidermolysis bullosa simplex (Takamori et al., 1985). Incubation of normal human skin with the blister fluid from patients with epidermolysis bullosa letalis, a severe and usually fatal congenital blister disease, results in dermal-epidermal separation. A number of common serine protease inhibitors prevented the separation (Matsumoto and Hashimoto, 1986).

Proteases are Associated with Inflammation. Proteases are important mediators and modulators of inflammation and have been demonstrated in non-blistering inflammatory disease states such as psoriasis and arthritis. The most abundant enzymes are the serine proteases elastase and cathepsin G (a chymotrypsin-like enzyme) from leukocytes; chymases (chymotrypsin-like enzymes), and tryptases (trypsin-like) enzymes from mast cells; plasminogen activator; and the metalloprotease collagenase from leukocytes. These enzymes are capable of cleaving a variety of connective tissue proteins including elastin, collagen, proteoglycans, and other basement membrane components.

Sulfur Mustard Induced Inflammatory Lesions Contain Proteases and Protease-Inhibitor Complexes. The proteolytic enzymes released upon exposure to sulfur mustard have not yet been isolated or characterized, but likely candidates include chymases and tryptases from mast cells, elastase and cathepsin G from leukocytes, plasminogen activator, and collagenase. Culture

fluids from mustard-induced inflammatory lesions in rabbit skin show 3 to 6 fold increased levels of proteases both in developing and healing lesions (Higuchi et al., 1987). These fluids will hydrolyze two synthetic peptide substrates, Boc-Leu-Gly-Arg-AFC (Boc = *t*-butyloxycarbonyl, AFC = 7-amino-4-trifluoromethyl coumarin) and Bz-Phe-β-naphthyl ester (Bz = benzoyl). The first peptide is a substrate for trypsin, tryptases, plasmin, plasminogen activator and other trypsin-like enzymes, while the latter is a substrate for chymotrypsin-like enzymes including chymases and cathepsin G. The rabbit skin culture fluids did not consistently hydrolyze four other synthetic peptide substrates (two for elastase and two for cathepsin G) or the protein elastin (elastase's natural substrate). Exposure of human skin in culture to sulfur mustard results in a 41 % increase in plasminogen activator activity (Dannenberg et al., 1989), an enzyme which is known to be associated with blister formation (Hashimoto et al., 1983). The enzymatic activity of chymases, tryptases, and angiotensin converting enzyme toward small synthetic substrates were not elevated.

The proteases found in the culture fluids from mustard-induced inflammatory lesions in rabbit skin are not present as free active enzymes, but are found as inactive complexes with their natural plasma protease inhibitors α_1-protease inhibitor and α_2-macroglobulin (Harada et al., 1987; Dannenberg et al., 1987; Higuchi et al., 1987). These complexes are formed as natural protein protease inhibitors from the plasma react with the proteases being released at the site of inflammation. The protease-inhibitor complexes are incapable of hydrolyzing protein substrates and complex formation thus protects the organism from further damage. The natural plasma protease inhibitors are probably not completely destroyed by exposure to low levels of sulfur mustard and are still available to react with some of the proteases released in the blister.

Protease-inhibitor complex formation hinders the identification of the proteases present in sulfur mustard induced culture fluid. Complexes of elastase and cathepsin G respectively with α_1-protease inhibitor and α_1-antichymotrypsin are inactive toward both synthetic peptide substrates and natural protein substrates. Thus the lack of hydrolysis of elastin or elastase substrates does not preclude the presence of inactivated elastase in the culture fluids. Similarly the lack of hydrolysis of the protein fibrin by the culture fluids doesn't exclude the presence of plasmin or plasminogen activator. In summary, it is now clear that there is a chymotrypsin-like enzyme (chymase), a trypsin-like enzyme (tryptase), and plasminogen activator in the sulfur mustard induced inflammatory lesions, but the presence of other enzymes has not been excluded.

Skin Serine Proteases Have Been Isolated and Characterized. The dermis of human skin is a rich source of mast cells and salt extraction of human skin has yielded two serine proteases, a chymase and a tryptase. These serine

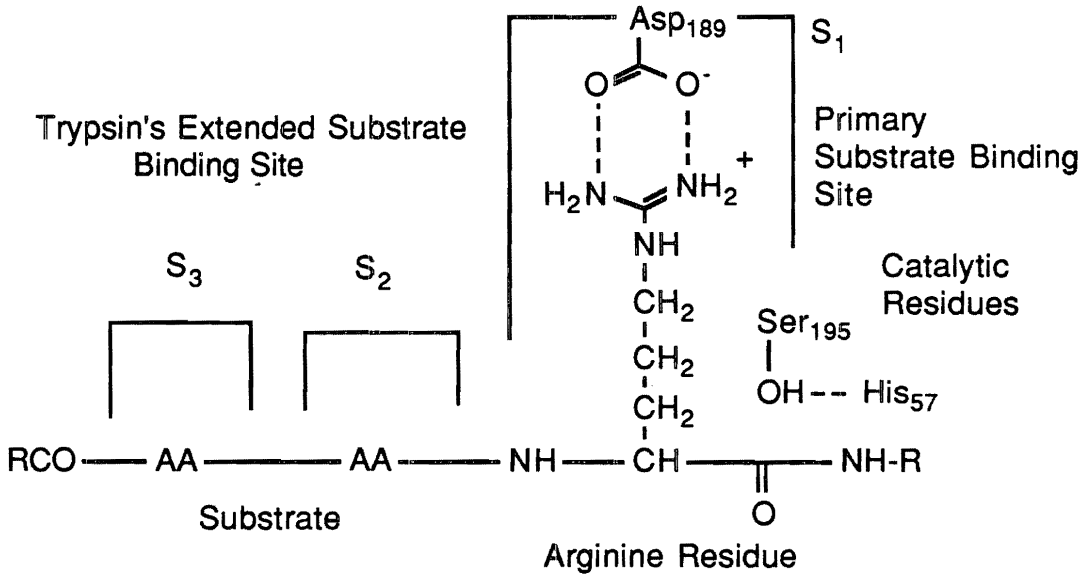
proteases are localized in the granule fraction of mast cells, a cell type which is located predominantly in connective tissue. The chymase has been demonstrated immunocytochemically to bind to the dermo-epidermal junction in skin (Sayama et al., 1987). Both the mast cell chymase and tryptase are able to specifically cleave proteins found in the dermal-epidermal boundary and cause vesication. The chymase is incompletely inhibited by plasma due to a 650 fold slower rate of reaction with the serpins α_1-protease inhibitor and α_1-antichymotrypsin (Schechter et al., 1989), while the tryptase appears not to be inhibited by most protein protease inhibitors (Schechter et al., 1983). This may explain the ready detection of chymase and tryptase activity in culture fluids from mustard-induced lesions in rabbit skin.

Rat mast cells contain two chymases (RMCP I and RMCP II) which have been more extensively characterized than the chymase from human skin. Both of these serine proteases are highly homologous to human mast chymase and human leukocyte cathepsin G. RMCP I and RMCP II have both been sequenced and the x-ray crystal structure of RMCP II has been determined, while the x-ray structure of RMCP I is underway (Woodbury and Neurath, 1980; Remington et al., 1988). The sequence of dog mast cell tryptase and a related dog mast cell protease have been determined by gene sequencing techniques (Vanderslice et al., 1989) and it is likely that the sequences for human skin tryptase and chymase will be available in the next few years.

The substrate specificity and inhibition profile of human skin chymase, human skin tryptase, RMCP I, RMCP II, and related enzymes have been studied in the laboratory of the principle investigator (Powers et al., 1985). Human skin chymase hydrolyzes peptide substrates containing aromatic amino acid residues and prefers Phe-AA and Tyr-AA bonds over Trp-AA bonds (AA = any amino acid residue) in contrast to chymotrypsin which prefers Trp over Phe and Tyr. One of the best peptide substrates is Suc-Phe-Val-Pro-Phe-NA (Suc = succinyl, NA = 4-nitroanilide). Human skin tryptase is a trypsin-like enzyme, but seems to prefer double basic residues in its substrates (Tanaka et al., 1983). For example, the thioester substrate Z-Lys-Arg-SBu-i (Z = benzyloxycarbonyl, SBu-i = thioisobutyl ester) is hydrolyzed by human skin tryptase with a k_{cat}/K_M = 59,000,000 $M^{-1}s^{-1}$, a second order rate constant which is close to the diffusion controlled rate.

Serine Protease Specificity. The specificity of serine proteases toward natural peptide substrates or synthetic inhibitors is determined by the nature of the primary substrate specificity pocket (S_1) and secondary subsites (S_2, S_3, etc.) on the surface of each individual enzyme. Trypsin's primary specificity site contains an Asp residue in the back of the S_1 pocket so that trypsin will only bind to and hydrolyze peptide substrates containing lysine or arginine residues (a schematic model of trypsin with a bound substrate is shown below). The

three-dimensional structure of chymotrypsin is quite similar except that the Asp-189 in trypsin is replaced by Gly-189 in chymotrypsin. As a result the S_1 pocket of chymotrypsin in very hydrophobic and chymotrypsin prefers substrates containing aromatic amino acid residues such as Trp, Tyr, and Phe. With many serine proteases, interactions of inhibitors with the extended substrate binding site (S_2, S_3, etc.) are important to increase the specificity and reactivity of the inhibitor. This is clearly the case with human skin chymase and tryptase. For example, interaction of the Lys in the substrate Z-Lys-Arg-SBu-i with the S_2 subsite of human tryptase results in an accelerated rate of hydrolysis, while little change in hydrolysis rate is observed with trypsin.



HYPOTHESIS

It is clear—no matter the exact mechanism of their release or their source—that proteases are major factors in the tissue destruction that accompanies mustard induced vesication. We propose that protease inhibitors will be effective antivesicants and should be useful both in preventing blistering and in the treatment of blisters. Appropriate target proteases are the mast cell chymase and tryptase, serine proteases which are localized in the skin and have the ability to cleave proteins at the dermal-epidermal junction. However other serine protease such as elastase and cathepsin G from leukocytes, and plasminogen activator may also be involved. Evidence for the involvement of other classes of proteases such as the metalloprotease collagenase or the thiolprotease cathepsin B is incomplete or lacking at present, although the mast cell tryptase is able to activate latent collagenase (Gruber et al., 1989).

Research Strategy. Since the exact target enzyme (or enzymes) is not known with certainty, we decided to synthesize general serine protease inhibitors, specific chymase inhibitors, specific tryptase inhibitors, specific plasminogen activator inhibitors, and specific inhibitors for other important serine proteases. During the last portion of this contract, we focused on inhibitors for trypsin-like enzymes since Smith et al. (1991) have recently reported increased hydrolysis rates for substrates of trypsin-like enzymes by mustard stimulated lymphocytes. Thus, these enzymes may be significant proteases involved in blistering.

PROGRESS REPORT

Research Goals

1. Prepare and submit for animal testing 3-5 inhibitors of serine proteases such as 3,4-dichloroisocoumarin and saccharins each year.

2. Prepare and submit for testing 3-5 inhibitors of human skin chymase each year.

3. Prepare and submit for testing 3-5 inhibitors of human skin tryptase each year.

4. Prepare and submit for testing each year 3-5 inhibitors for other human serine proteases-such as human leukocyte elastase, cathepsin G, and plasminogen activator-which may have a role in vesication.

5. Assay all inhibitors with human skin serine proteases and related enzymes for *in vitro* effectiveness.

Research Progress-Summary

1. A total of 45 serine protease inhibitors have been submitted in 3-5 g quantities for animal testing during the first two years of this contract. The structural classes include 9 isocoumarins, 5 saccharins, 6 peptide phosphonates, 3 benzoxazinones, 11 benzamidines, 5 derivative of *p*-guanidino benzoic acid and 6 miscellaneous compounds. All the inhibitors which were submitted are listed in the following table along with their sample numbers.

2. Various inhibitors have been surveyed for inhibitory potency against human neutrophil elastase, human neutrophil cathepsin G, rat skin tryptase, human skin tryptase, human skin chymase, human plasminogen activator, porcine pancreatic elastase, rat mast cell protease II, dog skin chymase, human lung tryptase and bovine chymotrypsin.

SAMPLES SUBMITTED: DMAD17-89-C-9008

BOT-NUM	WR-NUM	SUB-ID	RCHD	COMPOUND
ISOCOUMARINS (9 submitted)				
BL58572	268195	II-148	89/05/15	3,4-dichloroisocoumarin
BL57637	268119	II-134	89/03/20	3-chloroisocoumarin
BL57413	259666	II-137	89/03/13	4-chloro-3(2-phenylethoxy)isocoumarin
BM00482	268387	II-151	89/08/08	4-chloro-3-benzyloxyisocoumarin
BM04319	268693	MA-134	90/04/17	7-amino-4-chloro-3-cyclohexylmethoxyisocoumarin
BM04668	268715	GP1	90/05/17	4-chloro-3-methylisocoumarin
BM01096	268440	JO-138	89/09/12	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxy isocoumarin
BM00642	268397	II-152	89/08/21	4-chloro-3-(3-S-isothiureidopropoxy)isocoumarin
BM06288		GP2	90/09/05	4-chloro-3-methoxyisocoumarin
SACCHARINS (5 submitted)				
BL57977	268145	MA-84	89/04/19	N-benzoyl saccharin
BL57995	268147	MA-89	89/04/19	N-phenylacetyl saccharin
BL57986	268146	MA-87	89/04/19	N-diphenylacetyl saccharin
BL57931	268141	MA-86	89/04/19	N-furoyl saccharin
BM00464	268385	MA-96	89/08/01	N-cyanomethyl saccharin
PHOSPHONATES (6 submitted)				
BL57959	268143	II-22	89/04/19	Z-Met^{P}(OPh)$_2$
BL57968	268144	II-138A	89/04/19	Z-Val^{P}(OPh)$_2$
BL57422	259858	II-137A	89/03/13	Z-Phe^{P}(OPh)$_2$
BL57842	268132	II-139	89/04/10	Z-Phe-Phe^{P}(OPh)$_2$
BL59382	268241	II-147	89/06/14	Suc-Val-Pro-Phe^{P}(OPh)$_2$
BM04328	268694	III-3	90/04/17	Z-NHCH(AmPh)PO(OPh)$_2$

p-GUANIDINOBENZOIC ACID DERIVATIVES (5 submitted)

BM01363	268476	MA101	89/10/06	*p*-guanidino benzoic acid
BM01185	184335	MA-111	89/09/19	ethyl-*p*-guanidinobenzoate
BM02655	268570	MA-121	89/12/13	N(*p*-guanidinobenzoyl)valine amide
BM03143	268596	MA-115	90/01/23	O(*p*-guanidinobenzoyl)glycol amide
BM06304		MA-148	90/09/11	4-cyanophenyl *p*-guanidinobenzoate

BENZOXAZINONES (3 submitted)

BM00651	268398	JO-12	89/08/21	2-ethoxybenzoxazinone
BM05441		GP3	90/06/29	2-phenylamino-4H-3,1-benzoxazin-4-one
BM05807		GP4	90/07/24	2-benzylamino-4H-3,1-benzoxazin-4-one

BENZAMIDINES (11 submitted)

BM06804		GP5	90/11/02	1-(4-amidinophenyl)-3-phenylurea
BM06840		GP6	90/11/14	1-(4-amidinophenyl)-3-(4-chlorophenyl)urea
BM07481		GP7	90/12/18	1-(4-amidinophenyl)-3-benzylurea
BM07829		GP8	91/01/31	1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea
BM08004		GP9	91/02/19	(4-amidinobenzyl)benzyl ether
BM08184		GP10	91/03/08	bis(4-amidinophenyl)urea
BM08308		GP11	91/04/02	1-(3-amidinophenyl)-3-phenylurea
BM08595		GP12	91/04/23	(4-amidinobenzyl)phenylethyl ether
BM08764		GP13	91/05/05	1-(3-amidinophenyl)-3-(4-phenyoxyphenyl)urea
BM09903		GP14	91/08/05	(4-amidinobenzyl)-3-phenylpropyl ether
BM09912		GP15	91/08/05	(4-amidinobenzyl)-3-phenoxybenzyl ether

MISC. (6 submitted)

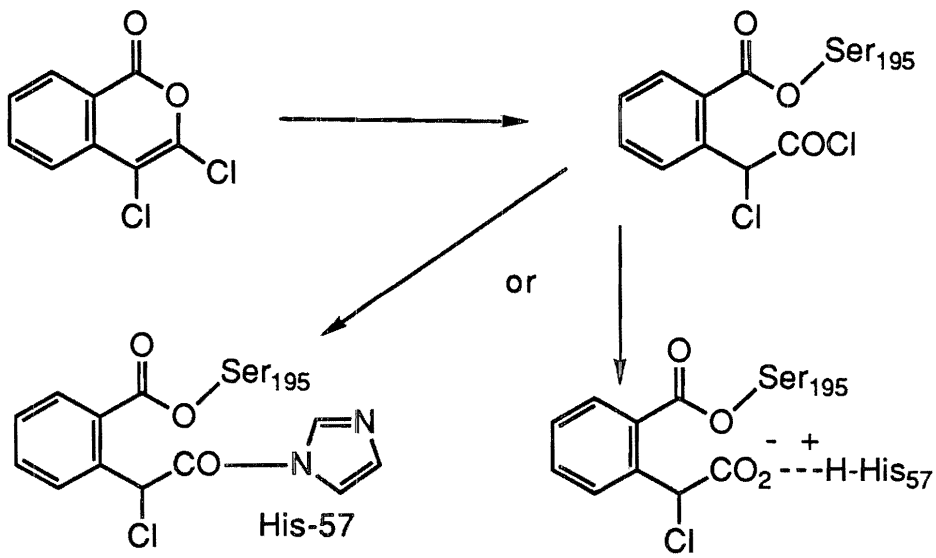
BL57646	015392	II-137B	89/03/20	isatoic anhydride
BL57940	268142	II-145	89/04/19	di(4-isovaleroylphenyl)sulfide
BM00491	099874	II	89/08/08	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide
BM07490		MA-60	90/12/18	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole
BM07838		MA-76	91/01/31	1-benzoyloxy-1,2,3-benzotriazole
BM07132		MA-68	90/11/27	N-(2-isothiureidoethyl)pthalimide

TOTAL SUBMITTED (8/30/91): 45

Inhibitors Submitted.

Isocoumarins-General Inhibitors. Dichloroisocoumarin (WR268195) is an excellent general inhibitor of serine proteases and was discovered in the laboratory of the principal investigator (Harper et al., 1985). With the exception of the bacterial enzyme subtilisin, 3,4-dichloroisocoumarin is an inactivator of all serine proteases which have been tested, including human leukocyte elastase, human skin chymase, dog skin chymase, rat mast cell protease I, and rat mast cell protease II.

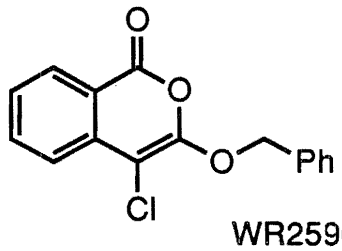
The dichloroisocoumarin ring system contains a masked acid chloride (or ketene) functional group which is exposed when an acyl enzyme is formed upon reaction with the active site serine of a serine protease (Harper et al., 1985). The acyl enzyme (top right of figure) which is formed initially can react further by acylating the active site histidine to form a doubly acylated enzyme derivative (bottom left) or can hydrolyze to form an acyl enzyme stabilized by a salt link between the protonated histidine and the inhibitor carboxyl group (bottom right). The monochloro derivative, 3-chloroisocoumarin (WR268119), inhibits chymotrypsin-like enzymes at slower rates than 3,4-dichloroisocoumarin and does not touch trypsin. The acyl enzymes formed upon reaction with dichloroisocoumarin have variable stabilities, but in general the half-lives for reactivation (deacylation) are greater than 8 hrs at pH 7.5.



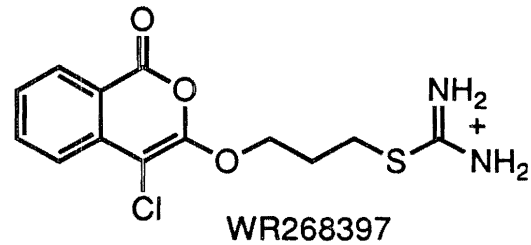
Dichloroisocoumarin and 3-chloroisocoumarin are formed by reaction of homophthalic acid with PCl_5.

Isocoumarins-Specific Inhibitors. We have also synthesized a number of isocoumarin inhibitors which are more specific for the active sites of chymases or tryptases.

Inhibitors targeted for chymase should contain an aromatic side chain which resembles the side chain of Phe, Tyr or Trp, while those inhibitors targeted for tryptase should containing a charged group which resembles the side chain of Arg or Lys. Several of the more specific isocoumarin inhibitors are shown below. Inhibitors with the benzyloxy (such as WR268387) or phenylethoxy groups (WR259666) were targeted at the chymases, while those with basic side chains (such as WR268397) were targeted at the tryptases.

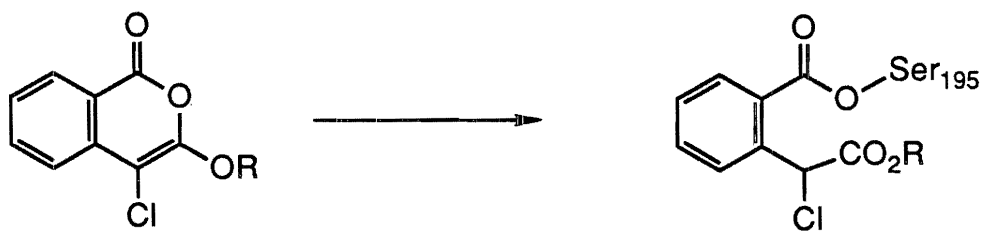


WR259666

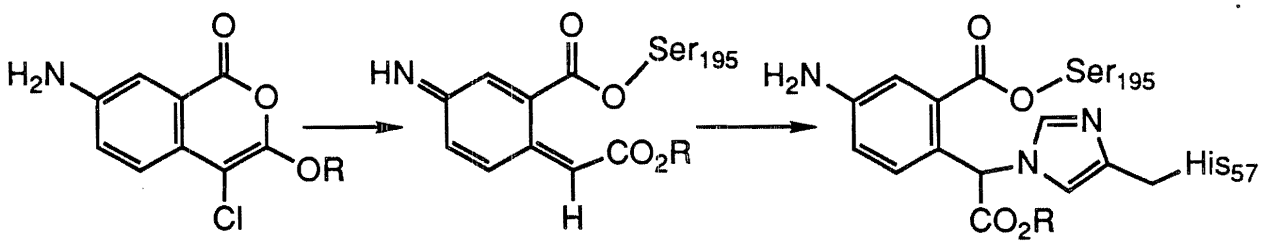


WR268397

The mechanism of inhibition of serine proteases by 3-alkoxy-4-chloroisocoumarins involves acylation of the active site serine-195 to form acyl enzymes with varying stabilities ($t_{1/2}$ = hrs to days) depending on the nature of the alkoxy group (Harper and Powers, 1985).

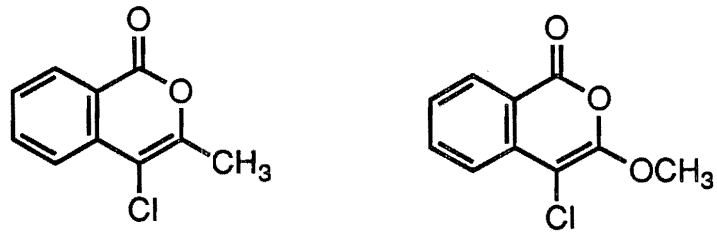


Analogous isocoumarins with electron donating substituents in the 7-position such as 3-alkoxy-7-amino-4-chloroisocoumarins (below) are mechanism-based or suicide inhibitors of serine proteases (Powers et al., 1989). These inhibitors also acylate serine proteases, but form stable acyl enzymes which are not reactivated upon long standing or upon treatment with hydroxylamine. The inhibition mechanism involves formation of an acyl enzyme which can then eliminate chloride to form a quinone imine methide (center). This intermediate then irreversibly alkylates His-57 with the formation of a stable covalent bond between enzyme and inhibitor. This mechanism is supported by x-ray crystallographic studies of complexes of isocoumarin inhibitors bound to the active site of porcine pancreatic elastase (Bode et al., 1989). Thus far, five separate isocoumarins have been studied crystallographically, two give simple acyl enzyme structures (above), two give acyl enzyme structures where His-57 has been alkylated, and one gives a mixture of both structures.

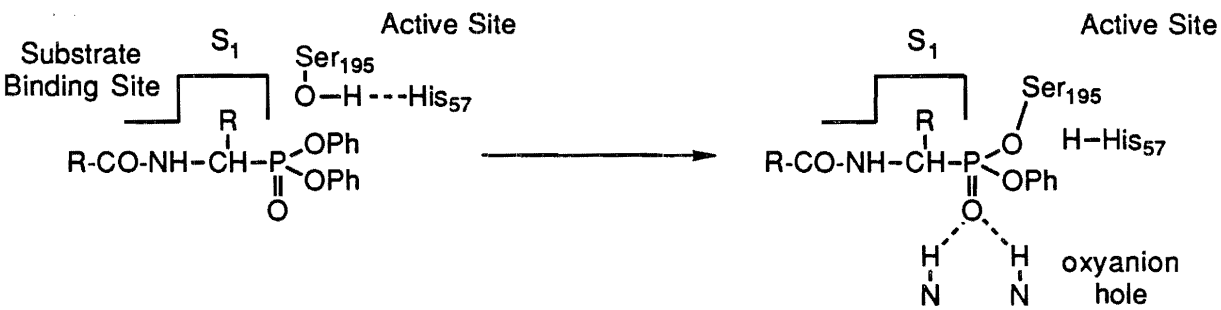


Isocoumarins which have been submitted and should inhibit serine proteases by the above mechanism include 7-amino-4-chloro-3-(cyclohexylmethoxy)isocoumarin (WR268693) and 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin (WR268440).

Two analogs of 3,4-dichloroisocoumarin are shown below. These two compounds were synthesized with the expectation that any isocoumarins with two small electronegative functional groups would be effective general serine protease inhibitors.

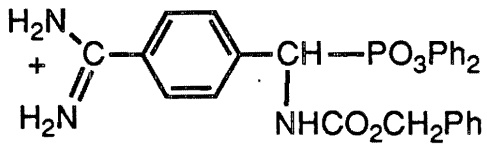


α-Aminoalkylphosphonates Diphenyl Esters. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations (Oleksyszyn and Powers, 1989; 1991). These peptide derivatives phosphonylate the active site serine to form stable phosphonyl derivatives. Good interactions with the S_1 pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative.

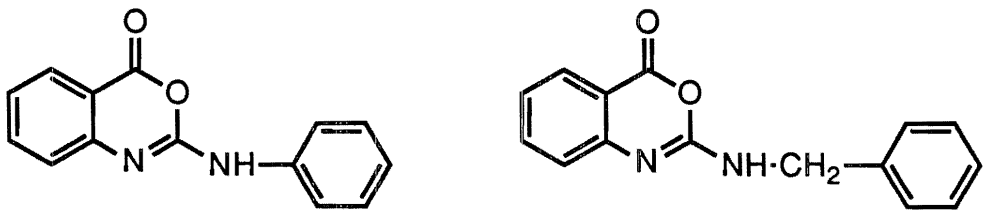


Phosphonate diphenyl ester inhibitors are chemically stable, relatively easy to synthesize, do not react with acetylcholinesterase, form very stable derivatives possibly due to their resemblance to the tetrahedral intermediate involved in peptide bond hydrolysis, and have considerable potential utility as therapeutic agents.

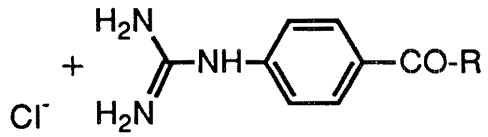
We have submitted a number of simple amino acid and peptide derivatives of phosphonate diphenyl esters including Z-Met^{P}(OPh)$_2$ (WR268143), Z-Val^{P}(OPh)$_2$ (WR268144), Z-Phe^{P}(OPh)$_2$ (WR259858), Z-Phe-Phe^{P}(OPh)$_2$ (WR268132), and Suc-Val-Pro-Phe^{P}(OPh)$_2$ (WR268241). More recently, we have accomplished the synthesis of the amidinophenyl phosphonate derivative (WR268694) shown below. The benzyloxycarbonyl (Z) derivative has been submitted recently for testing. We have also synthesized a few peptide derivatives of the amidinophenyl phosphonate, but not in sufficient quantities for submission.



Benzoxazin-4-ones. Substituted benzoxazin-4-ones were discovered to be potent inhibitors of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, cathepsin G, and chymotrypsin by the PI (Teshima et al., 1982). Mechanistic studies by Abeles showed that these compounds were forming stable acyl enzyme derivatives (shown below) with chymotrypsin (Hedstrom et al., 1984) and this has been confirmed by x-ray crystallographic studies with two benzoxazinones bound to PP elastase (Radhakrishnan et al., 1987). Due to the potential of benzoxazinone inhibitors of HL elastase for treatment of emphysema, a group at Syntex Canada has synthesized over 100 new benzoxazinones, carried out a structure-function study as substituents were varied on the ring system, and studied the plasma stability of these compounds (Spencer et al., 1986; Krantz et al., 1987; Krantz et al., 1990). We have submitted two benzoxazinones (BM05441 and BM05807) as elastase inhibitors.

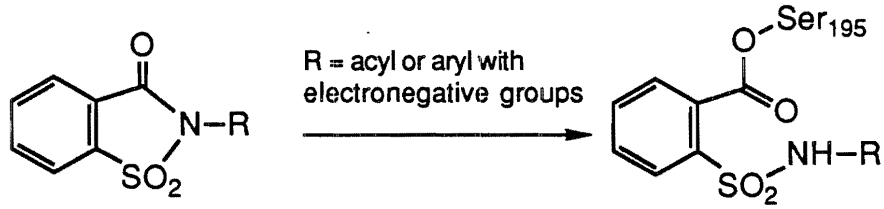


Guanidinobenzoic Acid Inhibitors. Esters of *p*-guanidinobenzoic acid have been reported to be potent inhibitors for various trypsin-like enzymes (Okutome et al., 1984; Fujii et al., 1977) and *p'*-nitrophenyl-*p*-guanidinobenzoate is widely used as an active-site titrant for these enzymes (Chase and Shaw, 1970). An active-site titrant for trypsin-like enzymes developed in the laboratories of the PI is benzyl *p*-guanidinothiobenzoate (Cook and Powers, 1983). We have submitted samples of five *p*-guanidinobenzoic acid derivatives for testing.

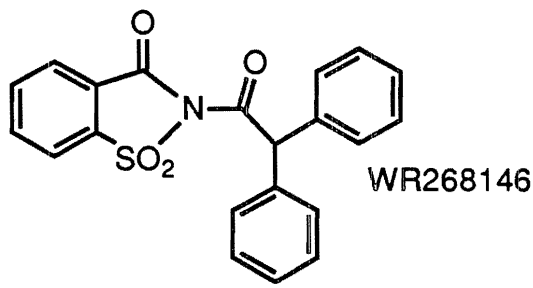


R = OH (WR268476)
OEt (WR184335)
NH-Val-NH_2 (WR268570)
OCH_2CONH_2 (WR268596)
C_6H_4-CN (BM06304)

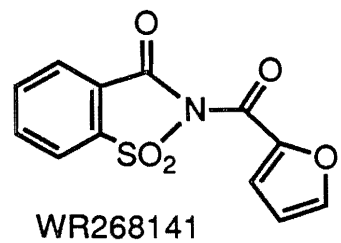
Saccharin Inhibitors. N-Acyl and N-aryl saccharins are potent acylating agents of HL elastase, cathepsin G, and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981). A few of the N-acyl derivatives such as N-furoyl, N-thienoyl, and N-benzoylsaccharin inhibit trypsin with IC_{50} values of 0.7-2.4 μM. These structures were initially designed as acyl transfer reagents, but studies using ^{35}S-labeled N-furoylsaccharin indicated that the saccharin portion of the inhibitor becomes covalently and stoichiometrically bound to both HL elastase and pancreatic elastase upon acylation.



Two of the saccharins which we have submitted are shown below.

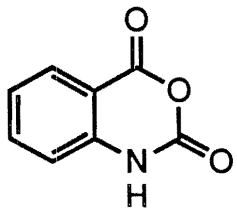


WR268146

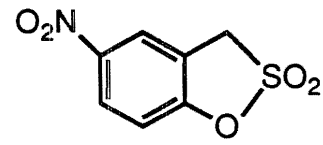


WR268141

Miscellaneous Inhibitors. Two heterocyclic general serine protease inhibitors have been submitted. They are isatoic anhydride which has been shown to acylate the active site of chymotrypsin and form a stable acyl enzyme (Moorman and Abeles, 1982) and 5-nitro-3H-1,2-benzoxathiole-2,2-dioxide which forms a stable sulfonyl derivative also with chymotrypsin.

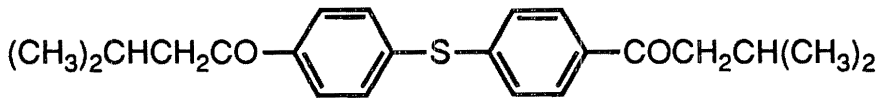


Isatoic Anhydride
WR015392



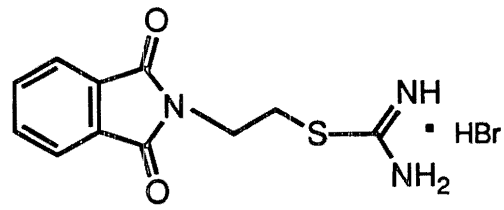
5-Nitro-3H-1,2-benzoxathiole-2,2-dioxide
WR099874

The sulfide shown below is an effective inhibitor of elastase which was discovered in the laboratory of the PI.

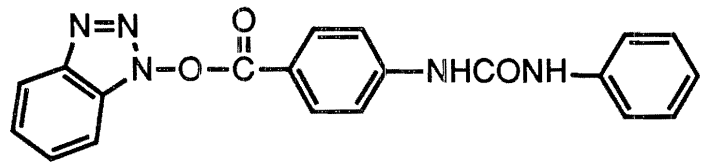


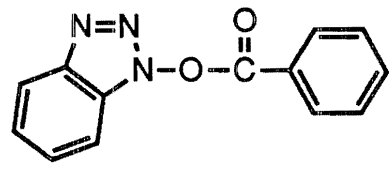
WR268142

The following phthalimide derivative was designed as a trypsin inhibitor.

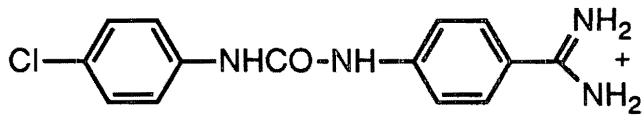


The following acyl derivatives of N-hydroxybenzotriazole inhibit trypsin.

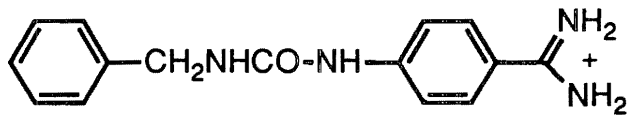




Benzamidine Inhibitors. Eleven benzamidine inhibitors have been submitted as reversible inhibitors for trypsin-like enzymes. These compounds interact with the S_1 pocket as well as other portions of the active site. Some representative examples are shown on the next page.



BM06840



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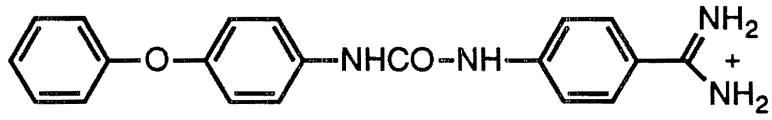

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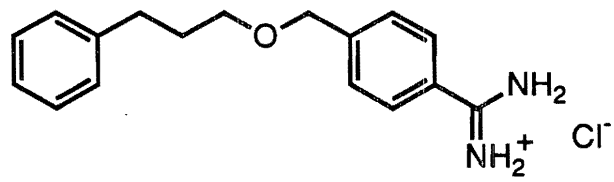

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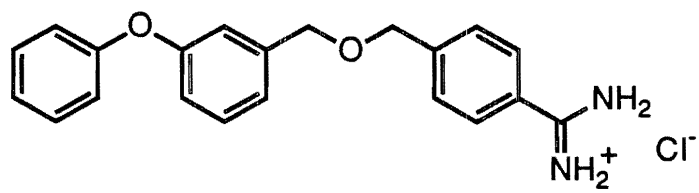


BM09912

Biological Test Data.

All the inhibitors which we have synthesized have been tested for inhibitory potency against a variety of serine proteases. Kinetic data obtained with the various inhibitors are shown in Tables I-III. Most of the inhibitors are irreversible or slowly reversible inhibitors and we report the second order inhibition rate constants $k_{obs}/[I]$. Several of the inhibitors reported in the tables have $k_{obs}/[I]$ values of 100,000 $M^{-1}s^{-1}$ or greater. A second order inhibition rate constant of this magnitude indicates that the reaction between equimolar concentrations of enzyme and the inhibitor is over in less than 0.2 min. (the time required for mixing the enzyme and inhibitor in the assays). The half-life of the inhibition reaction can be calculated from the equation $t_{1/2} = 0.693/\{[I]*k_{obs}/[I]\}$. Thus, an inhibitor with a $k_{obs}/[I]$ value of 100,000 $M^{-1}s^{-1}$ would have an inhibition half-life of 6.93 sec. at an inhibitor concentration of 1 μM, while an inhibitor with $k_{obs}/[I]$ = 10,000 would have a half-life of 69 sec. For the few reversible inhibitors investigated, K_I values (dissociation constant of the enzyme-inhibitor complex) or IC_{50} values are given.

The data with bovine chymotrypsin, cathepsin G, rat mast cell protease II, human skin chymase, and dog skin chymase is given in Table I. The best isocoumarin inhibitor in this table is 3-benzyloxy-4-chloroisocoumarin (WR268387) with a $k_{obsd}/[I]$ = 12,000 $M^{-1}s^{-1}$ for the human skin chymase. Increasing the length of the side chain at position 3 by one methylene group [4-chloro-3-(2-phenylethoxy)isocoumarin, WR259666] reduces the activity by a factor of 35.

The best phosphonate inhibitor for chymotrypsin-like enzymes is Suc-Val-Pro-Phe^{P}(OPh)$_2$, which corresponds to the sequence of an excellent 4-nitroanilide substrate for these enzymes. NMR studies with chymotrypsin indicate that only one of the two stereoisomers reacts with the enzyme ($k_{obsd}/[I]$ = 146,000 $M^{-1}s^{-1}$ calculated for the single isomer, the value in the table is for the DL mixture). The ^{31}P NMR of chymotrypsin inhibited by this peptide phosphonate shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonylated enzyme derivative (Oleksyszyn and Powers, 1991). The tripeptide phosphonate Suc-Val-Pro-Phe^{P}(OPh)$_2$ makes better interactions with the extended substrate binding site of the enzyme than is possible with the shorter dipeptide or amino acid phosphonate derivatives.

All of the saccharins submitted so far have high $k_{obsd}/[I]$ values with the various chymotrypsin-like enzymes and low IC_{50} values with the elastases tested. One of the better inhibitors in this family is N-furoylsaccharin. The acyl enzymes formed upon acylation of serine proteases by acyl saccharins have variable stabilities. Furoyl saccharin and benzoyl saccharin form inhibited elastase derivatives which are very stable and have half-lives for deacylation of 80-160 hrs. In contrast the

chymotrypsin derivatives have much shorter half-lives in the range of 1.9 hrs. One disadvantage of some acyl saccharins is their fairly rapid hydrolysis at neutral pH values.

The data with porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE) is given in Table II. All the isocoumarin derivatives reported in this table are excellent inhibitors of HLE, the best one being the 7-ureido derivative WR268440 with a $k_{obsd}/[I]$ = 140,000 $M^{-1}s^{-1}$ for HLE. It is also highly selective compared with 3,4-dichloroisocoumarin and 3-chloroisocoumarin which is a logical consequence of the improvement in binding resulting from substitution at the 7-amino group. We have also submitted an excellent benzoxazinone inhibitor for HLE (WR268398). Two analogs of this compound were poor inhibitors of the elastases, and were only moderate inhibitors of chymotrypsin-like enzymes.

The phosphonate derivatives which we have submitted thus far are not good inhibitors for PPE or HLE. This is not surprising since the sequence of the tripeptide was chosen for chymase inhibition and this sequence is very specific for the chymases as discussed earlier. An excellent phosphonate inhibitor for the elastases has been synthesized only in a small scale. This inhibitor, Boc-Val-Pro-Val^{P}(OPh)$_2$, has $k_{obsd}/[I]$ value of 27,000 $M^{-1}s^{-1}$ for human leukocyte elastase (HLE) and 11,000 $M^{-1}s^{-1}$ for porcine pancreatic elastase (PPE). Again this sequence corresponds to a good HLE substrate sequence and at low concentrations this peptide did not react with chymotrypsin.

The inhibition data obtained with bovine trypsin, human lung tryptase, rat skin tryptase, human skin tryptase, and human recombinant tissue plasminogen activator is given in Table III. The best tryptase inhibitor submitted is the 3-(isothioureidopropoxy)isocoumarin WR268397 with a $k_{obs}/[I]$ = 650,000 $M^{-1}s^{-1}$ for the rat skin tryptase, an extremely rapid inhibition rate. However, the acyl-enzyme formed with this inhibitor is unstable and the enzyme regains its activity within 5 min.

Several *p*-guanidinobenzoic acid derivatives have been tested as inhibitors for the various tryptases. One of these derivatives, O-(*p*-guanidinobenzoyl)glycolamide is an excellent inhibitor of the rat skin tryptase, but is a much poorer inhibitor of human skin tryptase. In contrast to the isocoumarin WR268397, the inhibited derivative did not regain enzyme activity upon standing. We pursued this lead and synthesized additional derivatives. The 4-cyanophenyl *p*-guanidinobenzoate was also a very potent inhibitor of trypsin and human lung tryptase. However it is probably not very stable in solution.

We then shifted our emphasis away from guanidino compounds to amidines when we learned that amidines are likely to be less toxic than guanidino derivatives. In the last few months of this contract we have been concentrating only on inhibitors for

tryptases since this may be the more important enzyme in blister formation. Aromatic benzamidine derivatives are reversible inhibitors for trypsin-like enzymes and we have been preparing a series of derivatives to see if we can increase their inhibitory potency for tryptases. The most potent inhibitor thus far for both trypsin and the human lung tryptase is 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea (GP8, BM07829) which has a K_I value of 1.6 μM with bovine trypsin and inhibits 71% of the activity of human lung tryptase at 226 μM.

Table I. Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Isocoumarin Inhibitors				
268195	3,4-dichloroIC[g]	570	28	580	27	82
268119	3-chloroIC[g]	330	24	85		
259666	4-chloro-3-(2-phenylethoxy)IC	3800	100	200	340	
268387	4-chloro-3-benzyloxyIC	32,000	220	3200	12,000	39,000
268693	7-amino-4-chloro-3-cyclohexylmethoxyIC				25	
268715	4-chloro-3-methyl IC	66	8			
BM06288	4-chloro-3-methoxy IC	206	25			
		Benzoxazinone Inhibitors				
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	77	reactivates			

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI			
	Miscellaneous Inhibitors					
015392	isatoic anhydride	580	114	250		
268142	di(4-isovaleroylphenyl)sulfide		NI[h]	25%[i]		
	Phosphonate Inhibitors					
268143	Z-Met^{P}(OPh)$_2$	1.6	1.1	24		
268144	Z-Val^{P}(OPh)$_2$	0.4	NI[h]	NI[h]		
259858	Z-Phe^{P}(OPh)$_2$	260	76	89		
268132	Z-Phe-Phe^{P}(OPh)$_2$	7.5	NI[h]	18		
268241	Suc-Val-Pro-Phe^{P}(OPh)$_2$	41,000	36,000	15,000	190,000	

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) ChyT[b]	Cathepsin G[c]	RMCP II[d]	Human Skin Chymase[e]	Dog Skin Chymase[f]
		Saccharin Inhibitors				
268145	N-benzoylsaccharin	15,000	45,000	16,000		
268147	N-phenylacetylsaccharin	11,000	31,000	1,300		
268146	N-diphenylacetylsaccharin	10,000	14,000	9,800		
268141	N-furoylsaccharin	22,000	39,000	20,000		
268385	N-cyanomethylsaccharin	NI[h]				

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 9 % Me_2SO at 25 °C. Enzyme concentrations were: chymotrypsin, 1.6 μM; cathepsin G, 0.8-1.6 μM; RMCP II, 38 nM; human skin chymase, 0.07 μM. Chymotrypsin and cathepsin G were assayed with Suc-Val-Pro-Phe-NA (0.5 μM), human skin chymase and RMCP II were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (88 μM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Inhibitor concentrations were in the range 5.2 μM-1000 μM. N-Cyanomethyl saccharin was inactive at 1 mM.

[c]Inhibitor concentrations were in the range: 4.8 μM-1000 μM.

[d]Inhibitor concentrations were in the range: 3-148 μM.

Table I (Continued). Inhibition of Bovine Chymotrypsin, Human Cathepsin G, Rat Mast Cell Protease II, Human Skin Chymase, and Dog Skin Chymase by Various Serine Protease Inhibitors.[a]

[e]Inhibitor concentrations were as follows: 4-chloro-3-(2-phenylethoxy)IC, 45 μM; 7-amino-4-chloro-3-cyclohexylmethoxyIC, 0.44 mM; Suc-Val-Pro-Phe$^{P}(OPh)_2$, 0.54 μM.

[f]Inhibitor concentrations were as follows: 3,4-dichloroisocoumarin, 540 μM; 3-benzyloxy-4-chloroIC, 1.0 μM.

[g]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

[h]No inhibition.

[i]Inhibition was not time dependent, and the % inhibition was measured at 92 μM.

Table II. Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Isocoumarin Inhibitors		
268195	3,4-dichloroIC[d]	2,500	9,000
268119	3-chloroIC[d]	510	3,900
259666	4-chloro-3-(2-phenylethoxy)IC		
268440	4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyIC	59	140,000
268715	4-chloro-3-methyl IC	NI[e]	72%[f]
BM06288	4-chloro-3-methoxy IC	601	87
	Miscellaneous Inhibitors		
015392	isatoic anhydride		
268142	di(4-isovaleroylphenyl)sulfide		2 μM[g]
099874	5-nitro-3H-1,2-benzoxathiole-2,2-dioxide		50
	Benzoxazinones		
268398	2-ethoxy-4H-3,1-benzoxazin-4-one		110,000
BM05441	2-phenylamino-4H-3,1-benzoxazin-4-one	216	NI
BM05807	2-benzylamino-4H-3,1-benzoxazin-4-one	NI	NI

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) PPE[b]	HLE[c]
	Saccharin Inhibitors		
268145	N-benzoylsaccharin	5.2 μM[h]	2.4 μM[h]
268147	N-phenylacetylsaccharin		
268146	N-diphenylacetylsaccharin		
268141	N-furoylsaccharin	0.58 μM[h]	0.36 μM[h]
268385	N-cyanomethylsaccharin		
	Phosphonate Inhibitors		
268143	Z-Met^{P}(OPh)$_2$	NI[e]	0.8
268144	Z-Val^{P}(OPh)$_2$	2.5	90
259858	Z-Phe^{P}(OPh)$_2$	NI	6
268132	Z-Phe-Phe^{P}(OPh)$_2$	NI	NI
268241	Suc-Val-Pro-Phe^{P}(OPh)$_2$	NI	NI

[a]Inactivation rates were measured by an incubation method in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer, containing 8-12 % Me_2SO at 25 °C. Enzyme concentrations were: PPE, 1.6 μM; HLE, 0.3 μM. PPE was assayed with Suc-Ala-Ala-Ala-NA (0.75 mM), and HLE was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.5 mM).

[b]Inhibitor concentrations were in the range: 3-1000 μM.

[c]Inhibitor concentrations were in the range: 1.2-1000 μM.

[d]Data was obtained from Harper, J. W., Hemmi, K., and Powers, J. C. (1985) Biochemistry **24**, 1831-1841.

Table II (Continued). Inhibition of Porcine Pancreatic Elastase (PPE) and Human Leukocyte Elastase (HLE) by Various Serine Protease Inhibitors.[a]

[e]No inhibition.

[f]Inhibition was not time dependent.

[g]Inhibition was not time dependent and the IC_{50} was obtained.

[h]IC_{50} values obtained from Zimmerman, M., Morman, H., Mulvey, D., Jones, H, Frankshun, R. and Ashe, B. M. (1980) J. Biol. Chem. **255**, 9848-9851.

Table III. Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Isocoumarin Derivatives					
268195	3,4-dichloroisocoumarin	200	190	610		70
268397	4-chloro-3-(3-isothioureido-propoxy)isocoumarin	46,000	260,000	650,000	83,000	13,000
	Guanidinobenzoic Acid Derivatives					
268476	*p*-guanidinobenzoic acid	NI[g]				
184335	ethyl *p*-guanidinobenzoate	4.3	1.7	0.7		NI[g]
268570	N-(*p*-guanidinobenzoyl) valine amide	4.4	4.7	1.3	2,000	
268596	O-(*p*-guanidinobenzoyl) glycolamide	100	19%[h]	130,000	5.8	NI[g]

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
BM06304	4-cyanophenyl *p*-guanidinobenzoate	150,000	91,000			
	Phosphonate Inhibitors					
268694	Z-NHCH(AmPh)PO_3Ph_2[i]	2,000		16		
	Benzamidine Derivatives	K_I (μM)	% Inhibition[j]			
BM06804	1-(4-amidinophenyl)-3-phenylurea	23	41			
BM06840	1-(4-amidinophenyl)-3-(4-chlorophenyl)urea	18	51			
BM07481	1-(4-amidinophenyl)-3-benzylurea	19	49			
BM07829	1-(4-amidinophenyl)-3-(4-phenoxyphenyl)urea	1.6	71			
BM08004	(4-amidinobenzyl)benzyl ether	47	31			
lBM08184	bis(4-amidinophenyl)urea	8				

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

WR Compound No.	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
BM08308	1-(3-amidinophenyl)-3-phenylurea	50				
BM08595	(4-amidinobenzyl)phenylethyl ether	14				
BM08764	1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea	10				
BM09903	(4-amidinobenzyl)-3-phenylpropyl ether	16				
BM09912	(4-amidinobenzyl) 3-phenoxybenzyl ether	20				

Table III (Continued). Inhibition of Bovine Trypsin, Human Lung Tryptase, Rat Skin Tryptase, Human Skin Tryptase, and Human Recombinant Tissue Plasminogen Activator by Isocoumarins, Derivatives of *p*-Guanidinobenzoic Acid, Phosphonate Inhibitors, Benzamidines, and Miscellaneous Inhibitors.

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				
WR Compound No.	Inhibitor	Bovine Trypsin[b]	Human Lung Tryptase[c]	Rat Skin Tryptase[d]	Human Skin Tryptase[e]	Human r-t-PA[f]
	Miscellaneous Inhibitors					
BM07490	1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole	14,000	600			
MA-76	1-benzoyloxy-1,2,3-benzotriazole	1,100				
BM07132	N-(2-isothiureidoethyl)pthalimide	NI				

[a]Inactivation rates were measured by an incubation method. Enzyme concentrations were: trypsin, 0.12 μM; rat skin tryptase, 0.015 μM; human skin tryptase, 0.12 μM and 0.02 μM; human r-*t*-PA, 0.017 μM Bovine trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM). Human lung tryptase, human skin tryptase and rat skin tryptase were assayed with Z-Arg-SBzl·HCl (0.07 mM) in the presence of 4,4'-dithiodipyridine (0.33 mM).

[b]Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 1μM; *p*-guanidinobenzoic acid, 0.44 mM; ethyl *p*-guanidinobenzoate, 0.43 mM; *p*-guanidinobenzoyl valine amide,0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 mM; 4-cyanophenyl *p*-

Table III (Continued).

guanidinobenzoate, 0.46 μM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 1.22 μM; 1-benzoyloxy-1,2,3-benzotriazole, 22 μM.

[c]Conditions were as follows: 0.1M Hepes, 0.5M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureido-propoxy)isocoumarin, 0.42 μM; ethyl *p*-guanidinobenzoate, 0.42 mM; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.42 mM; 4-cyanophenyl *p*-guanidinobenzoate, 0.22 μM; 1-(4-(phenylureido)benzoyloxy)-1,2,3-benzotriazole, 11.6 μM.

[d]Conditions were as follows: 25 mM phosphate, 0.5M NaCl, 1mM EDTA, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.44 μM; ethyl *p*-guanidinobenzoate, 0.45 mM ; *p*-guanidinobenzoyl valine amide, 0.42 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.44 μM.

[e]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 0.35 μM; *p*-guanidinobenzoyl valine amide, 3.5 μM; O-(*p*-guanidinobenzoyl)glycolamide, 1.7 mM.

[f]Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5 and 8-12% Me_2SO at 25 °C. Inhibitor concentrations were as follows: 4-chloro-3-(3-isothioureidopropoxy)isocoumarin, 4.3 μM; ethyl *p*-guanidinobenzoate, 0.44 mM; O-(*p*-guanidinobenzoyl)glycolamide, 0.43 mM.

[g]No inhibition.

[h]Inhibition was not time dependent and the % inhibition was measured at 0.42 mM.

[i] AmPh = 4-amidinophenyl

[j]Inhbitior concentrations were 226 μM.

Animal Testing Priorities

The various compounds which we have submitted have been prioritized for animal testing and the following table lists the priority which we have assigned to each compound and the reasons for that priority.

High-1 WR268195 3,4-dichloroisocoumarin
This isocoumarin is a general serine protease inhibitor and effectively inhibits most of the enzymes tested.

High-2 GP8 1-(amidinophenyl)-3-(4-phenoxyphenyl)urea
This benzamidine should be very stable in solution and is an excellant competitive inhibitor of trypsin-like enzymes.

High-3 WR268241 Suc-Val-Pro-Phe^{P}(OPh)$_2$
This peptide phosphonate is a reactive and specific inhibitor for chymotrypsin-like enzymes including chymases.

High-4 WR268397 4-chloro-3-(3-isothioureidopropoxy)isocoumarin
This isocoumarin is a very reactive inhibitor for the rat skin tryptase.

High-5 WR268398 2-ethoxy-4H-3,1-benzoxazin-4-one
This benzoxazinone inhibitor is an effective inhibitor for elastase.

High-6 WR268141 N-furoylsaccharin
This saccharin is a general protease inhibitor and inhibits elastases and chymotrypsin-like enzyme quite effectively and is probably a moderate inhibitor for trypsin-like enzymes.

High-7 BM06304 4-cyanophenyl *p*-guanidinobenzoate
This guanidinium substituted derivative is an effective inhibitor for trypsin-like enzymes

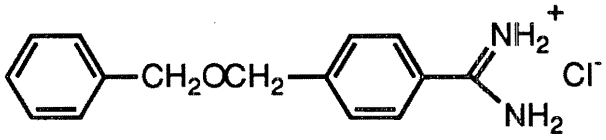
High-8 WR268440 4-chloro-7-(N-phenylcarbamoyl)amino-3-propoxyisocoumarin
This isocoumarin is an effective elastase inhibitor.

High-9 WR268142 di(4-isovaleroylphenyl)sulfide
This aromatic derivative is an effective elastase inhibitor.

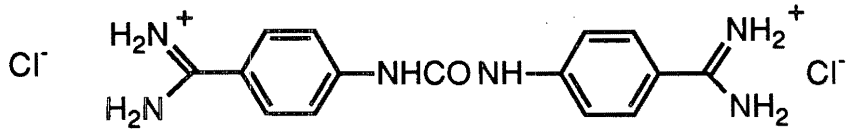
High-10 WR268387 4-chloro-3-benzyloxyisocoumarin
This isocoumarin is an effective inhibitor for chymotrypsin-like enzymes.

SYNTHESIS

Benzamidines. Six new benzamidines were synthesized in the final six months of the project We successfully overcame the initial difficulties in the synthesis of these class of compounds and submitted a total of eleven benzamidines. The last compounds submitted are (4-amidinobenzyl) benzyl ether hydrochloride (**GP 9**), bis(4-amidinophenyl)urea dihydrochloride (**GP 10**), 1-(3-amidinophenyl)-3-phenylurea hydrochloride (**GP 11**), (4-amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**), 1-(3-amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**), (4-amidinobenzyl)-3-phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**). The structures are shown below.



GP 9, BM08004



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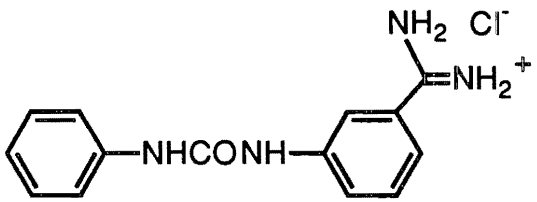
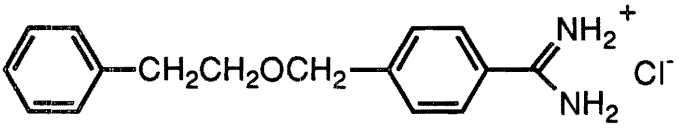
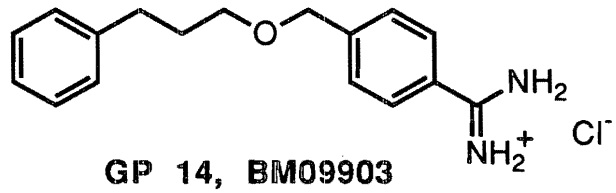


GP 11, BM08308

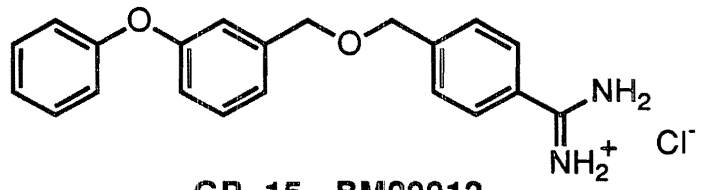


GP 12, BM08595

GP 13, BM08764



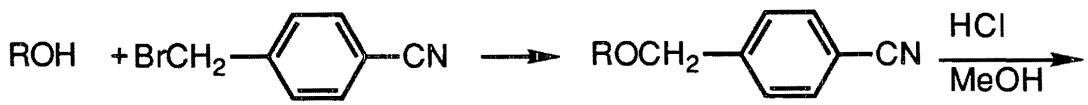
GP 14, BM09903

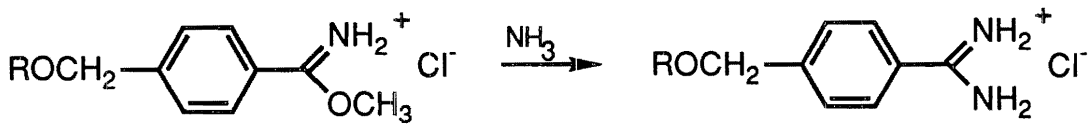


GP 15, BM09912

Synthetic Schemes. (4-Amidinobenzyl)benzyl ether (**GP 9**) was synthesized from the cyano ether obtained by the reaction of sodium benzyloxide and α-bromo-*p*-tolunitrile. The cyano ether was converted to the imidate ester by treating it with dry methanol in the presence of HCl. The imidate ester on reacting with ammonia gave the desired amidino compound.

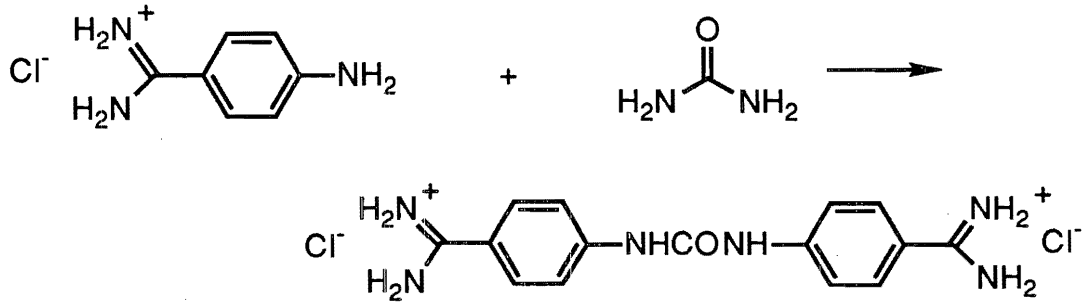
(4-Amidinobenzyl)phenylethyl ether hydrochloride (**GP 12**) was prepared by replacing benzyl alcohol with phenylethyl alcohol and keeping the rest of the procedure as in **GP 9**.





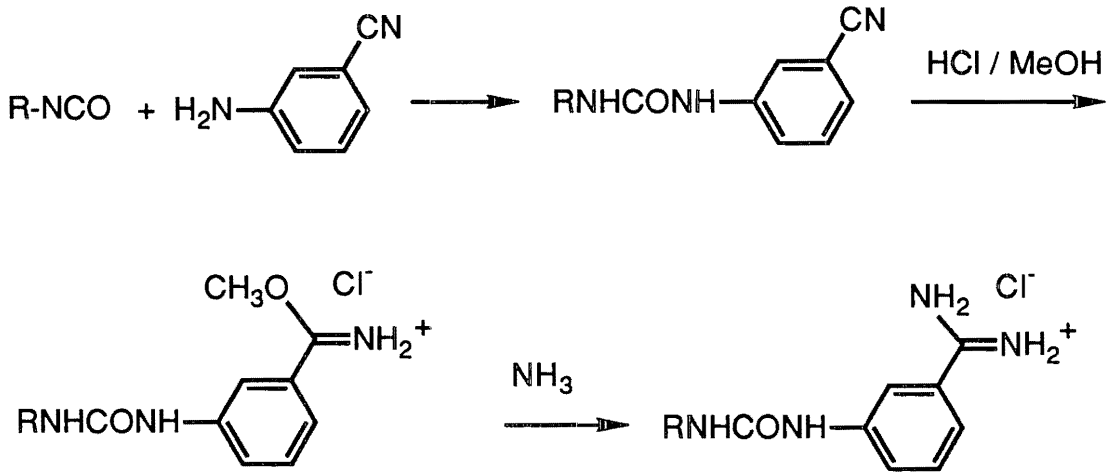
(4-Amidinobenzyl)phenylpropyl ether hydrochloride (**GP 14**) and (4-amidinobenzyl)3-phenoxybenzyl ether hydrochloride (**GP 15**) were also synthesized using the same method with ROH being phenylpropyl alcohol and 3-phenoxybenzyl alcohol respectively.

Bis(4-amidinophenyl)urea dihydrochloride (**GP 10**) was made by condensing 4-aminobenzamidine dihydrochloride with urea.



1-(3-Amidinophenyl)-3-phenylurea hydrochloride (**GP 11**) was prepared starting from 3-aminobenzonitrile. Phenyl isocyanate and 3-aminobenzonitrile were condensed in refluxing benzene and the resulting urea was converted to the imidate ester by the treatment with dry methanol in presence of dry HCl. Refluxing the imidate ester in dry isopropanol saturated with ammonia afforded the desired amidino compound.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (**GP 13**) was prepared using the procedure described above except that phenyl isocyanate was replaced with 4-phenoxyphenyl isocyanate.



(4-Amidinobenzyl)benzyl ether hydrochloride (GP 9). A solution of benzyl alcohol (1.08 g, 0.01 mol) in dry THF (10 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (1.96 g, 0.01 mol) in dry THF (10 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.41 g, 44 %); mp 102-104 °C. ^{1}H NMR (DMSO-d_6) δ: 4.58 (s, 2H); 4.65 (s, 2H); 7.25-7.40 (m, 5H); 7.59 (d, 2H); 7.84 (d, 2H); 9.23 (bs, 2H); 9.41 (bs, 2H). Anal. Calcd. for $C_{15}H_{17}Cl_1N_2O$: C, 65.10; H, 6.19 Cl, 12.81; N, 10.12. Found: C, 64.98; H, 6.20; Cl, 12.73; N, 10.02.

Bis(4-amidinophenyl)urea dihydrochloride (GP 10). A suspension of urea (0.60 g, 0.01 mol) and 4-aminobenzamidine (4.16 g, 0.02 mol) in water (5 mL) was heated under reflux for 24 h. The condenser was then removed and the heating further continued for 24 h. The reaction was then trichurated with water (25 mL). The solid was filtered out dissolved in water and the clear solution acidified with HCl to get the product as a white solid

(3.1 g, 84 %); mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 7.68 (d, 4H); 7.84 (d, 4H); 9.00 (s, 4H); 9.23 (d, 4H); 10.45(s, 2H). Anal. Calcd. for $C_{15}H_{18}Cl_2N_6O \cdot 0.3\ H_2O$: C, 48.07; H, 4.96; Cl, 18.96; N, 22.43. Found: C, 48.06; H, 5.05; Cl, 19.03; N, 22.55.

1-(3-Amidinophenyl)-3-phenylurea hydrochloride (GP 11). To a stirred solution of 3-aminobenzonitrile (4.72 g, 0.04 mol) in benzene (100 mL) was added phenyl isocyanate (5.00 g, 0.042 mol) and the reaction refluxed for 5 h and further stirred at room temperature overnight. The separated white solid was filtered out and recrystallised from methanol (8.2 g, 84 %).

Dry HCl was passed through a cooled solution of the cyano urea (4.00 g, 0.016 mol) and dry methanol (2.7 g, 0.08 mol) in dry dimethoxyethane (100 mL) for 2 h and the reaction stored in a refrigerator for 14 h. Solvent was then evaporated under reduced pressure and the imidate ester obtained, as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (4 g) was dissolved in dry isopropanol saturated with ammonia (200 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. Solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white solid of the title compound (2.1 g, 55 %); mp 252-254 °C. ^{1}H NMR (DMSO-d_6) δ: 6.98 (t, 1H); 7.20-7.35 (m, 3H); 7.40-7.55 (m, 3H); 7.70 (d, 1H); 7.95 (s, 1H); 9.1 (bs, 2H); 9.32 (bs, 2H); 9.45 (bs, 1H); 9.18 (bs, 1H). Anal. Calcd. for $C_{14}H_{15}Cl_1N_4O$: C, 57.83; H, 5.20 Cl, 12.19; N, 19.27. Found: C, 57.74; H, 5.20; Cl, 12.16; N, 19.21.

(4-Amidinobenzyl)phenylethyl ether hydrochloride (GP 12). This compound was prepared using the procedure described for compound **GP 9** and replacing benzyl alcohol with phenyl ethyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 2.88 (t, 2H); 3.69 (t, 2H); 4.60 (s, 2H); 7.15-7.30 (m, 5H); 7.49 (d, 2H); 7.80 (d, 2H); 9.17 (bs, 2H); 9.37 (bs, 2H). Anal. Calcd. for $C_{14}H_{14}Cl_2N_4O$: C, 66.09; H, 6.59; Cl, 12.19; N, 9.63. Found: C, 66.18; H, 6.61; Cl, 12.09; N, 9.56.

1-(3-Amidinophenyl)-3-(4-phenoxyphenyl)urea hydrochloride (GP 13). This compound was prepared using the

procedure described for compound **GP 11** and substituting 4-phenoxyphenyl isocyanate for phenyl isocyanate. ^{1}H NMR (DMSO-d_6) δ: 6.91-7.20 (m, 4H); 7.10 (t, 1H); 7.30-7.40 (m, 3H); 7.45-7.58 (m, 3H); 7.70 (d, 1H); 7.95 (s, 1H); 9.00 (s, 2H); 9.34 (s, 1H); 9.36 (s, 2H); 9.55 (s, 1H). Anal. Calcd. for $C_{20}H_{19}Cl_1N_4O_2 \cdot 0.75$ H_2O: C, 60.60; H, 5.17; Cl, 8.96; N, 14.14. Found: C, 60.62; H, 5.19; Cl, 8.96; N, 14.05.

(4-Amidinobenzyl)-3-phenylpropyl ether hydrochloride (GP 14). A solution of 3-phenylpropyl alcohol (2.72 g, 0.02 mol) in dry THF (20 mL) was added slowly to sodium hydride (2.4 g, 0.10 mol) and the suspension stirred at room temperature for 15 min. To this stirred suspension was then added dropwise a solution of α-bromo-*p*-tolunitrile (3.92 g, 0.02 mol) in dry THF (20 mL) and the reaction stirred at room temperature for 24 h. Excess sodium hydride was destroyed by careful addition of water and the reaction taken up in ethyl acetate. The organic layer was washed successively with dil. HCl, water and brine, dried over anhydrous magnesium sulfate and solvent evaporated to get the cyano ether as a liquid.

Dry HCl was passed through a cooled solution of the cyano ether (1.00 g, 0.004 mol) and dry methanol (0.67 g, 0.020 mol) in dry dimethoxyethane (30 mL) for 1 h and the reaction stored in a refrigerator for 16 h. Solvent was then evaporated under reduced pressure and the imidate ester, obtained as a yellow solid, was stored in a vacuum desiccator over KOH for 24 h.

The imidate ester (1 g) was dissolved in dry isopropanol saturated with ammonia (50 mL) and the reaction refluxed for 6 h and further stirred overnight at room temperature. The solvent was evaporated and the solid obtained was recrystallized from 2N HCl to get white crystals of the title compound (0.295 g, 31 %); mp 76-78 °C. ^{1}H NMR (DMSO-d_6) δ: 1.84 (m, 2H); 2.62 (t, 2H); 3.45 (t, 2H); 4.55 (s, 2H); 7.10-7.90 (aromatic, 9H); 9.20 (bs, 2H); 9.45 (bs, 2H). Anal. Calcd. for $C_{17}H_{21}Cl_1N_2O$: C, 66.99; H, 6.94 Cl, 11.63; N, 9.19. Found: C, 67.05; H, 6.95; Cl, 11.54; N, 9.20.

(4-Amidinobenzyl)-3-phenoxylbenzyl ether hydrochloride (GP 15). This compound was prepared using the procedure described for compound **GP 14** and replacing 3-phenylpropyl alcohol with 3-phenoxylbenzyl alcohol. ^{1}H NMR (DMSO-d_6) δ: 4.55 (s, 2H); 4.63 (s, 2H); 6.95-7.80 (aromatic, 13H); 9.16 (bs, 2H); 9.40 (bs, 2H). Anal. Calcd. for $C_{21}H_{21}Cl_1N_2O_2$: C, 68.38; H, 5.74; Cl, 9.61; N, 7.59. Found: C, 68.20; H, 5.78; Cl, 9.48; N, 7.44.

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